Development of novel antiviral drugs to combat human pathogenic arenaviruses

Thesis submitted for the degree Candidata pharmaciae to the
Department of Microbiology, School of Pharmacy,
Faculty of Mathematics and Natural Sciences, University of Oslo
2006

Anette Tølløfsrud Gundersen
The Scripps Research Institute, Department of Immunoloy
10550 North Torrey Pines Road
La Jolla, California 92037

TABLE OF CONTENTS

ACKNOWLEDGEMENTS……. iii
ABSTRACT…….. ix
A. **INTRODUCTION**……..... 1
A.1 Arenaviruses…….. 1
A.1.1 Classification of arenaviruses…….. 1
A.1.2 Structure, genome and proteins of arenaviruses……... 2
A.1.3 The GP of arenaviruses mediate host cell attachment and entry…….. 4
A.1.4 Alpha-dystroglycan is the cellular receptor for arenaviruses……... 5
A.2 Lassa fever represents a severe threat for human health and a major humanitarian problem... 6
A.3 Current therapy against arenaviruses……. 7
A.4 Targeting arenavirus receptor binding and entry is a promising new anti-viral strategy…….. 9
A.5 Small molecule inhibitors of protein interactions derived from combinatorial chemical libraries…….. 10
B. **MATERIALS AND METHODS**…… 13
B.1 Reagents and antibodies…… 13
B.2 Cell lines…… 13
B.3 Virus strains, purification and quantification…… 13
B.4 Detection of LCMVGP in ELISA……. 14
B.5 Immunoblotting……. 14
B.6 Molecular biology techniques…… 15
B.6.1 Polymerase chain reaction (PCR)……. 15
B.6.2 Phenol chloroform extraction……... 15
B.6.3 Agarose gel electrophoresis…….. 16
B.6.4 Gel extraction…… 16
B.6.5 Treatment of DNA fragments with calf intestinal phosphatase (CIP)…….. 17
B.6.6 Ligation of DNA fragments with T4 DNA ligase…… 17
B.6.7 Transformation of *E.coli.*……..... 17
B.6.8 Plasmid DNA purification using the QIAprep spin miniprep kit……. 18
B.7 Generation of pseudotyped retroviral vectors…… 18
B.8 Infection of human cells with retroviral pseudotypes…… 19
B.9 Steady-Glo® luciferase assay…… 19
B.10 Screening of chemical libraries: inhibition of the infection with retroviral pseudotypes by compounds from combinatorial chemical libraries……... 20
B.11 Target-specificity of compound mixtures…… 21
B.12 Determination of the dose-response characteristics of selected candidate compounds……... 21
B.13 Blocking of pseudotype infection with sulfated polysaccarides…….. 22
B.14 Intracellular FACS staining for LCMV-NP using mAb 113……... 23
B.15 Detection of LCMV-NP by immunofluorescence staining……. 23
C. **RESULTS**……. 25
C.1 Discovery of novel small molecule inhibitors of Lassa fever virus (LFV) infection from combinatorial chemical libraries……... 25
C.1.1 Production and characterization of retroviral pseudotypes……... 25
C.1.2 Screening of combinatorial chemical libraries……. 28
C.1.3 Determination of the target specificities of candidate compounds……... 29
C.1.4 Validation of candidate compounds in different human and primate cells…….. 30
C.1.5 Dose-response curves of candidate compounds……... 31

C.1.6 Determination of the activity of candidate compounds against other human pathogenic arenaviruses…… 31
C.2 Discovery of novel small molecule inhibitors of Lassa fever virus (LFV) infection from natural product libraries……. 35
C.2.1 Screening of natural product libraries……... 35
C.2.2 Deconvolution of compound mixtures……. 36
C.3 Evaluation of polyaninonic compounds as anti-arenaviral drugs…….. 38
C.3.1 Selection of candidate polymers: heparin, HS, DS and fucoidan……. 38
C.3.2 Test of heparin, HS, DS, fucoidan and polymer X for anti-viral activity against arenaviruses…… 39
C.3.3 What is the role of HS and glycosaminoglycans in arenavirus infection?..... 41
C.3.4 Evaluation of polymer X as a drug against LCMV…….. 43
C.4 Production of recombinant Lassa fever virus glycoprotein……... 47
C.4.1 Design of the LFVGP-foldon fusion protein…… 49
C.4.2 Expression and characterization of the GP1-foldon fusion proteins…… 50
D. **DISCUSSION**……... 51
D.1 Discovery of novel small molecule inhibitors of Lassa fever virus (LFV) infection from combinatorial chemical libraries and natural products libraries……... 51
D.2 Evaluation of polyanionic compounds as anti-arenaviral drugs…… 53
D.3 Production of recombinant Lassa fever virus glycoprotein……... 54
D.4 Outlook…….. 55
D.4.1 Further validation of candidate compounds……. 55
D.4.2 Determination of the mechanism of action of candidate drug…….. 56
D.4.3 Extension of small molecule screening to other drug targets and other emerging pathogens…… 56
E. **REFERENCES**……. 58

ACKNOWLEDGEMENTS

I would like to thank the following people:

Stefan Kunz, my tutor at TSRI, for his enthusiasm regarding my project, his guidance and expertise, and for all his help when making the figures and writing my thesis.

Professor Tor Gjøen, the supervisor of my master thesis at the University of Oslo, for good support and for providing me the opportunity to come to The Scripps Research Institute.

Dr. Michael B. A. Oldstone, the head of the Viral-Immunobiology laboratory at TSRI for his generous support, Ms. Jillian M. Rojek (TSRI) for technical and scientific advice, Dr. Dale Boger and Dr. Jin Wei (TSRI) for the chemical libraries, Dr. Kevin P. Campbell (Howard Hughes Medical Institute, University of Iowa), Dr. Juan-Carlos de la Torre (TSRI), and Dr. Michael Buchmeier (TSRI) for materials. The retroviral construct pLZRs-Luc-gfp was kindly provided by Dr. Gary Nabel.

For this work, I received a stipend from “S.G. Sønnelands Foundation” and a loan from “Statens Lånekasse”. My laboratory expenses at TSRI were covered by US Public Health grant AI55540 (M.B.A.O., S.K.), and grant 1U54 AI065359 of the Pacific Southwest Regional center of Excellence for Emerging Infectious Disease (S.K.).

ABSTRACT

Arenaviruses merit significant attention both as powerful models to study viral pathogenesis and as important human pathogens. Lymphocytic choriomeningitis virus (LCMV) infection of its natural host, the mouse, represents a powerful experimental system that provided novel concepts in immunology and virology that have been extended to other viruses, bacteria, and parasites. With over 200, 000 infections per year and several thousand deaths, Lassa virus (LFV) is by far the most important among the human pathogenic arenaviruses and represents a severe threat for human health. There are no licensed arenavirus vaccines and the current therapies are not optimal. Therefore, it is important to develop better antiviral drugs to combat the threats of arenavirus infections.

A fundamental reason for the high mortality of infections with human pathogenic arenaviruses is a failure of the host's immune system to control viral replication, leading to an unchecked viremia associated with hemorrhagic disease. Since rapid dissemination of the virus critically depends on attachment and entry into host cells, drugs targeting these steps will give the host's immune system a wider window of opportunity for the generation of an efficient anti-viral immune response. The goal of my research was therefore the development of novel anti-viral drugs that are able to block these initial steps of infection. In a first approach to identify such "gatekeeper" drugs I performed high-throughput small molecule screening using combinatorial chemical libraries, which represent a powerful technology for discovery of specific inhibitors of receptor-ligand interactions. In a collaborative effort with the laboratory of Dr. Dale Boger (Department of Chemistry, Scripps) I used a high-throughput screening assay for inhibitors of LFV infection using retroviral pseudotypes containing LFVGP in their envelope and a luciferase reporter gene. My screening identified several small molecule compounds that show specific blocking of LFV infection in several human and primate cell types with IC50 values in the range of 1-10 μM. Some of the candidates were also active against retroviral pseudotypes the South American hemorrhagic fever viruses Junin, Machupo, and Guanarito.

In a second approach, I evaluated the activity of anionic polymers like heparin, dextrane sulfate, and fucoidan, previously shown to have anti-viral effects, against human pathogenic arenaviruses. In contrast to published data, I found no significant activity of heparin and heparan sulfate against arenavirus infection and could demonstrate that heparan sulfate and other glycosaminoglycans are generally not involved in arenavirus infection. Dextrane sulfate and fucoidan indeed showed some anti-viral activity, however, they were far less efficient than claimed by other researchers. This motivated me to start to evaluate novel polymer drugs for activity against arenaviruses.

A. INTRODUCTION

Viral infectious diseases have threatened humankind throughout our existence. Although advances in medical science have changed the demographics of mortality with an increase in life expectancy to more than seventy-five years in the developed world, viral infections are still rampant in underdeveloped countries with life expectancies of less than 40 years in the poorest nations. Beyond contributing to mortality, viral infections also leave affected individuals often physically and mentally disabled, exacerbating human suffering and the socio-economic impact caused by these diseases.

A.1 Arenaviruses

A.1.1 Classification of arenaviruses

Two major groups of arenaviruses are currently recognized: the Old World arenaviruses with lymphocytic choriomeningitis virus (LCMV) and Lassa fever virus (LFV) as representatives and the larger group of the New World arenaviruses, which are divided into three major Clades, A, B, and C (Buchmeier et al., 2001). LCMV infection of its natural host, the mouse, represents a powerful experimental system that provided novel concepts in immunology and virology that have been extended to other viruses, bacteria, and parasites (Buchmeier et al., 2001; Oldstone, 2002). LCMV is also a prevalent human pathogen and congenital infections of the central nervous system by LCMV are considered an important problem in human pediatric medicine.

Arenaviruses are also the causative agents of several severe hemorrhagic fevers (HF) with high mortality in humans (Buchmeier et al., 2001; Geisbert and Jahrling, 2004). Lassa fever is the second most important human viral HF after Dengue and affects an estimated 180 million individuals living in its endemic regions in Western Africa. With over 200, 000 infections per year and several thousand deaths, LFV represents a severe threat for human health and a major humanitarian problem (McCormick and Fisher-Hoch, 2002). There is currently neither an efficient cure nor an efficacious vaccine for this disease and survivors are often left with substantial neurological impairment. Among the New World arenaviruses, Junin virus causes Argentine HF, and is a serious public health problem in Argentina. Machupo virus causes Bolivian HF, and Guanarito and Sabia virus have emerged as etiological agents of severe HF in Venezuela and Brazil, respectively. Infections with these arenaviruses in humans result in high mortality of 15 to 30%. Apart from the severe humanitarian

burden caused in endemic regions, air travel regularly imports arenavirus HF cases into metropolitan areas around the globe, placing local populations at risk.

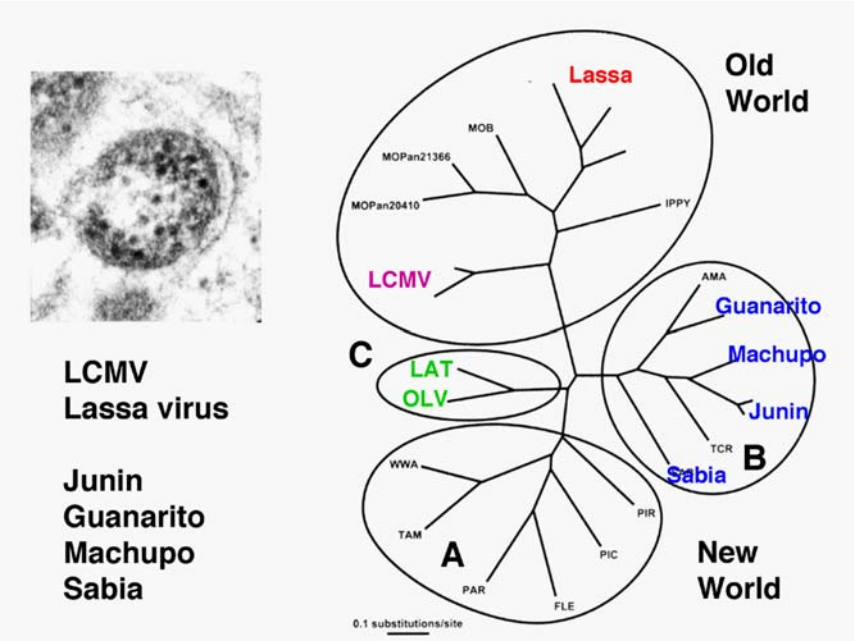

Figure A1: phylogenetic tree of arenaviruses. Left: electron microscopic image of a typical arenavirus particle and a list of the human pathogenic arenaviruses. Right: Phylogenetic tree of the arenavirus family, displaying the major subgroups and Clades.

A.1.2 Structure, genome and proteins of arenaviruses

Arenaviruses are enveloped viruses with a bisegmented negative single-stranded RNA genome and a life cycle restricted to the cell cytoplasm. Virions are pleomorphic but typically spherical with an average diameter of 90 to 110 nm, and are covered with surface glycoprotein spikes. Virions enclose the genomic RNAs as helical ribonucleoprotein structures in circular configurations. Virus particles have a “sandy” appearance from which the family name is derived.

Each genomic RNA segment, L (ca 7.3 kb) and S (ca 3.5 kb), uses an ambisense coding strategy to direct the synthesis of two polypeptides in opposite orientation, separated by an intergenic region (IGR) with a predicted folding of a stable hairpin structure (Fig. A2). The S RNA encodes the viral glycoprotein precursor, GPC, (ca 75 kDa) and the nucleoprotein, NP, (ca 63 kDa), whereas the L RNA encodes the viral RNA dependent RNA polymerase (RdRp, or L polymerase) (ca 200 kDa), and a small RING finger protein Z (ca 11 kDa). The NP and L coding regions are transcribed into a genomic complementary mRNA, whereas the GPC and Z coding regions are not translated directly from

genomic RNA, but rather from genomic sense mRNAs that are transcribed using as templates the corresponding antigenome RNA species, which also function as replicative intermediates. The LCMV mRNAs have extra non-templated nucleotides (nt) and a cap structure at their 5'-ends, but the origin of both the cap and 5'-non-templated nt extensions remain to be determined. Transcription termination of subgenomic non-polyadenylated viral mRNAs has been mapped to multiple sites within the distal side of the IGR, suggesting that the IGR acts as a bona fide transcription termination for the virus polymerase. A widely accepted model for the control of Arenavirus RNA replication and gene transcription proposes that at early times of infection, low levels of NP prevents the virus polymerase to go across the IGR, and hence transcription is favored over replication. Increasing intracellular NP levels during the course of the infection would unfold secondary RNA structures within the IGR. This would attenuate structure dependent transcription termination at the IGR, thus promoting replication of genome and antigenome RNA species.

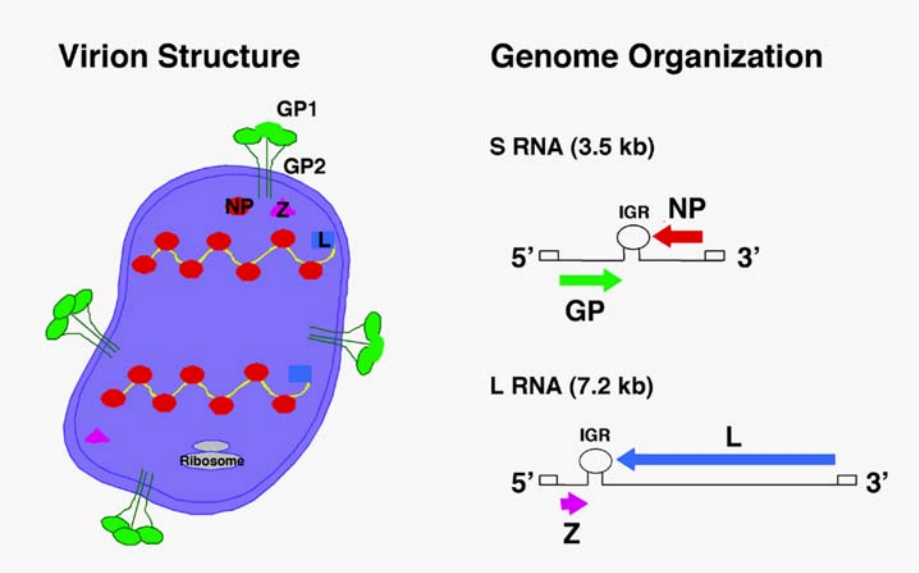

Figure A2: Schematic of arenavirus particles and their genome organization. For details see text. (Image provided by Dr. Juan-Carlos de la Torre, Scripps Research Institute).

A.1.3 The GP of arenaviruses mediates host cell attachment and entry

The arenavirus glycoprotein precursor GPC is coded in the S segment and synthesized as a single polypeptide chain. GPC is then post-translationally cleaved by the cellular protease SKI-1/S1P cellular protease to yield the two mature virion glycoproteins GP-1 (40-46 kDa) and GP-2 (35 kDa) (Lenz et al., 2001; Beyer et al., 2003; Kunz et al., 2003). GP-1 is located at the top of the spike, away from the membrane, and is held in place by ionic interactions with the N-terminus of the transmembrane GP-2, that forms the stalk of the spike (Fig. A3) GP-1 is the virion attachment protein that mediates virus interaction with host cell surface receptors.

Upon receptor binding, arenavirus virions are internalized by uncoated vesicles and released into the cytoplasm by a pH-dependent membrane fusion step (Borrow et al., 1994). The pH-dependent fusion between viral and cell membranes in the acidic environment of the endosome is mediated by the GP2 portions of arenavirus GPs.

Based on their structure-function relationship, viral proteins mediation membrane fusion have been classified to belong to either class I or class II fusion proteins. The class I fusion proteins are translated at GP precursor proteins, which are proteolytically processed to give rise to metastable trimers consisting of GP1/GP2 heterodimers. Whereas the peripheral head subunit mediates receptor binding, the transmembrane subunit is responsible for membrane fusion itself. Destabilization sets off a series of conformational rearrangements within the transmembrane subunit leading to the formation of the post-fusion six-helix bundle conformation. This state is characterized by C-terminal α-helices packing into the hydrophobic grooves of a trimeric coiled-coil core, formed by N-terminal α-helices (Fig. A3). Thereby, the viral envelope and the target membrane are drawn together to give rise to the fusion pore. Cross-linking experiments carried out with purified LCMV indicated that the GP-1/GP-2 heterodimers would form tetramers, which were partly disulfide linked among their GP-1subunits (Burns and Buchmeier, 1991). However, more recent work clearly demonstrated the presence of two alpha-helices separated by a glycosylated peptide loop within the ectodomain of arenavirus GP2 forming a trimeric pre-fusion complex (Gallaher et al., 2001; Eschli et al., 2006) (as shown in Fig.A3).

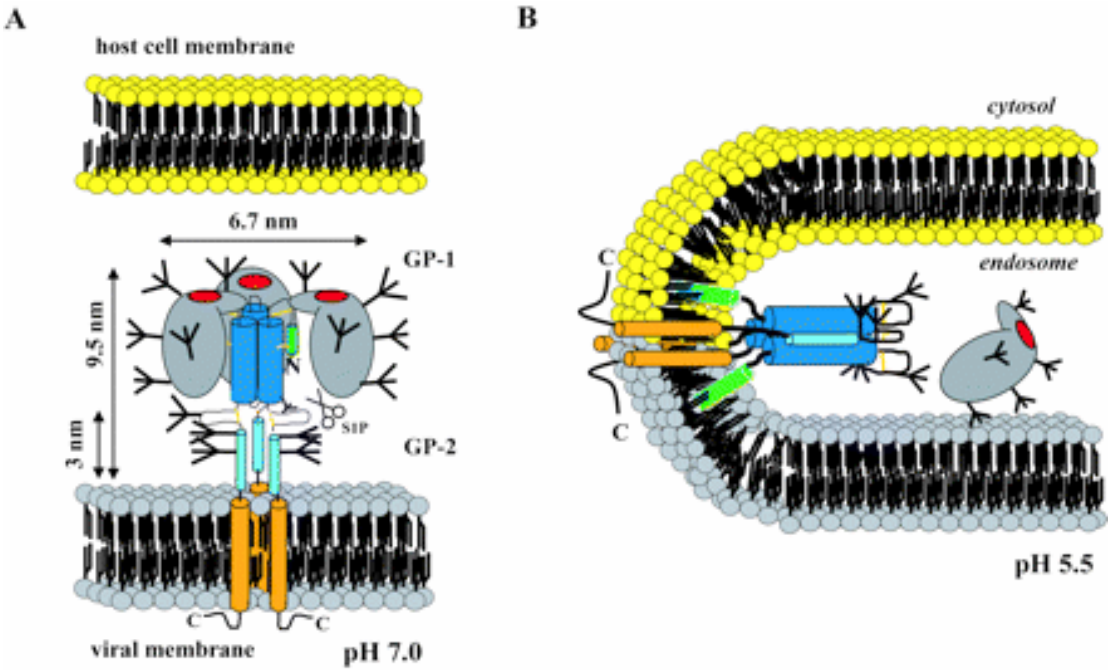

Figure A3: Proposed class I model of the arenavirus GP. (A) Arenavirus GP in its hypothetical pre-fusion state. GP-1 forms the globular head subunit, whereas GP-2 forms the membrane fusion-mediating subunit. GP-1 is covered by a dense carbohydrate shield, with the exception of the receptor binding site, depicted in red. (B) GP spike in its hypothetical low-energy post-fusion state, after formation of the viral fusion pore. The membrane of the infected host cell is colored in yellow and the viral membrane in light gray. The GP-2 subunit is shown in its predicted six-helix bundle conformation; thus, the α-helix 2 packs in an anti-parallel fashion into the grooves of the N-terminal coiled-coil core. Taken from Eschli et al., (2006).

A.1.4 Alpha-dystroglycan is the cellular receptor for arenaviruses

The first cellular receptor for LFV, LCMV and Clade C New World arenaviruses is α-dystroglycan (α-DG) (Cao et al., 1998; Spiropoulou et al., 2002), the extracellular part of dystroglycan (Fig. A4). DG was initially identified as the central component of the dystrophin-glycoprotein complex (DGC) in the membrane of skeletal muscle (Barresi and Campbell, 2006). In vertebrates, DG is encoded as a single protein, which is cleaved into α-DG, a peripheral protein, and β-DG, a membrane protein (Fig. A4A). DG is expressed in most developing and adult tissues and provides a molecular link between the extracellular matrix (ECM) and the actin-based cytoskeleton (Fig. A4B).

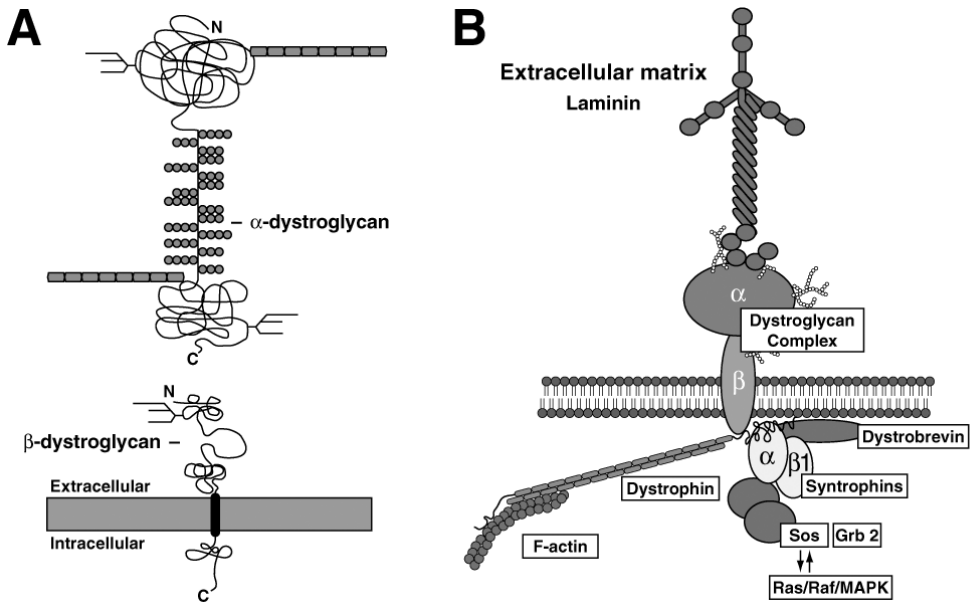

Figure A4: Schematic representation of DG (A) and a DG complex with the ECM protein laminin (B). (modified from Kevin Campbell, Howard Hughes Medical Institute, University of Iowa).

A.2 Lassa fever represents a severe threat for human health and a major humanitarian problem

Lassa fever is the second most important human viral HF after Dengue and affects an estimated 180 million individuals living in its endemic regions in Western Africa. With over 200, 000 infections per year and several thousand deaths, LFV represents a severe threat for human health and a major humanitarian problem (McCormick and Fisher-Hoch, 2002; Geisbert and Jahrling, 2004). There is currently neither an efficient cure nor an efficacious vaccine for this disease and survivors are often left with substantial neurological impairment.

LFV was first isolated in 1969 after a hospital outbreak in northern Nigeria and is classified within the Old World arenavirues. The natural reservoir of Lassa fever virus (LFV) is the rodent *Mastomys natalensis* and LF is endemic in Sierra Leone, Liberia, and Nigeria, where it represents a major cause of death. The fatality rate of LF in hospitalized patients is >15%, rising to more than 50% in some outbreaks. In recent years, air travel resulted in the import of LF cases into Europe, the United States, Japan, and Canada, placing local populations at risk.

LF has an insidious onset and presents with a wide spectrum of clinical manifestations, making diagnosis based on clinical symptoms often difficult. The incubation period of LF is 7-18 days, followed by fever, weakness and general malaise. A majority of patients develop cough, headache,

sore throat, and gastrointestinal manifestations. Signs of increased vascular permeability such as facial edema and pleural effusions indicate a poor prognosis. In lethal cases, deterioration is rapid with progressive signs and symptoms of pulmonary edema, respiratory distress, and shock, accompanied by bleeding from mucosal surfaces. A highly predictive factor for disease outcome is the extent of viremia. Patients developing a fatal LFV infection have higher viral loads and are unable to limit viremia while survivors have lower viral load and clear the virus.

The host's control of LFV replication is primarily mediated by cellular immunity. Antibodies play a modest role in acute LFV infection as patients can recover in the absence of a neutralizing antibody response. Like in other human viral HF, hallmark of fatal LF in humans is a marked immunosuppression (Geisbert and Jahrling, 2004).

Despite recent progress in the development of a LF vaccine (Fisher-Hoch, 2004; Geisbert et al., 2005), to my knowledge, to date no human vaccine trials have taken place. The only drug currently available for treatment of acute LF is the nucleoside analogue ribavirin, which significantly reduces mortality (McCormick et al., 1986). Since ribavirin does not neutralize free virus, it is less efficient in patients with high virus loads, especially late in infection. The strong predictive value of virus concentration in blood for a disastrous disease outcome in LF indicates a close competition between virus spread and the anti-viral immune response. **Rapid viral dissemination critically depends on efficient attachment of the virus to receptor molecules on target cells and subsequent entry. Drugs able to block virus-receptor binding and/or entry will therefore give the host's immune system an advantage by providing a wider window of opportunity for the generation of an efficient anti-viral immune response. The development of novel anti-viral drugs targeting these early steps of infection appears therefore as a promising approach for better treatment of Lassa fever in humans.**

A.3 Current therapy against arenaviruses

The only licensed drug against arenaviruses, which is currently available is the guanosine analogue ribavirin (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide) (Parker, 2005). In vitro and in vivo studies have documented the prophylactic and therapeutic value of ribavirin against several arenaviruses. Ribavirin has been shown to reduce significantly both morbidity and mortality in humans associated with LFV infection (McCormick et al., 1986). As with other nucleoside analogues, ribavirin is a prodrug that needs to be converted to nucleotide metabolites to exert its antiviral activity. Ribavirin

is phosphorylated by adenosine kinase to ribavirin monophosphate (RMP), which is then converted into its triphosphate form (RTP) by the successive action of mono-and di-phosphate kinases. Ribavirin can exerts its antiviral activity by a variety of mechanisms. RMP is a strong inhibitor of IMP dehydrogenase, which causes a large reduction in the intracellular levels of GTP and dGTP that can affect viral RNA synthesis. On the other hand RTP can interfere with the capping of viral mRNA, and with the function of the virus RNA-dependent RNA polymerase (RdRp). Recent evidence indicates that RTP can be efficiently used as substrate by the RdRp of some riboviruses, which results in C to U and G to A transitions. This mutagenic activity of RTP has been linked to its antiviral activity via lethal mutagenesis. The precise mechanisms by which ribavirin interferes with arenavirus multiplication remain to be determined. One of the problems associated with the use of ribavirin is that in a high percentage of cases (> 40%) treated individuals develop haemolytic anemia. Likewise, ribavirin has been associated with congenital disorders and hence it should be not used with pregnant women. In addition, oral ribavirin appears to be significantly less effective than the one administered intravenously, which pose some additional logistic complications for its use in regions with limited clinical infrastructure.

Several ribavirin related inhibitors of IMP dehydrogenase, including ribamidine (1-beta-D-ribofuranosyl-1,2,4-tiazole-3-carboxamide) (Andrei and de Clercq, 1993), as well as acyclic and carbocyclic adenosine analogue inhibitors of the S-adenosylhomocysteine (SAH) hydrolase (Andrei and de Clercq, 1990) have been show to have also anti-arenaviruses. Likewise, phenotiazines compounds (Candurra et al., 1996), myristic acid (Cordo et al., 1999), several disulfide-based compounds (Garcia et al., 2002), and brassinosteroids (Castilla et al., 2005) have been reported to have activity against several arenaviruses. **However, apart from ribavirin, none of these compounds have been tested in human trials and their efficacy in vivo in currently unknown.**

Another class of compounds that showed significant anti-viral activity against arenaviruses are sulfated polysaccharides like dextrane sulfate, fucoidan, heparan sulfate, and heparin (Andrei and de Clercq, 1990). These anionic polymers have anti-viral activity against a number of human pathogenic viruses, including HIV (Kilby and Eron, 2003) and have been studied extensively in the past decade. Based on their large size and charged nature, these polymer drugs are thought to function as inhibitors of cell attachment and entry. In case of HIV, a direct interaction of the polymer drugs with specific sites of the viral GP has been demonstrated recently.

In case of arenaviruses, the therapeutic anti-viral potential of these drugs and their mechanism of action are still controversial. One goal of the present study is therefore a systematic analysis of the anti-viral activity against human pathogenic arenaviruses of candidate anionic polymer drugs and the

elucidation of their mechanism of action using a panel of state-of-the art tools and assays available in the laboratory.

A.4 Targeting arenavirus receptor binding and entry is a promising new anti-viral strategy

The strong predictive value of virus concentration in blood for a disastrous disease outcome in human arenavirus infection indicates a close competition between virus spread and the anti-viral immune response. Rapid viral dissemination critically depends on efficient attachment of the virus to receptor molecules on target cells and subsequent entry. Drugs able to block virus-receptor binding and/or entry will therefore give the host's immune system an advantage by providing a wider window of opportunity for the generation of an efficient anti-viral immune response. The development of novel anti-viral drugs targeting these early steps of infection appears therefore as a promising approach for better treatment of arenavirus infection in humans.

Since binding of a virus to its cellular receptor(s) is the initial step of viral infection, it provides a primary target for therapeutic intervention. The feasibility of this type of approach has been demonstrated in the efforts to develop drugs against human immunodeficiency virus (HIV-1) (reviewed by Eron and Kilby, 2003). In the late 1980s, recombinant forms of the HIV receptor CD4 have been generated that proved to be potent inhibitors for laboratory strains of HIV but failed to block infection with primary isolate. The recombinant receptor-derived drug PRO542 is a tetramer hybrid that contains the receptor domains of CD4 in a human IgG backbone and is efficacious in blocking the receptor binding-site on GP120 of diverse strains of HIV-1. In pilot studies, anti-viral activity was detected in adults and in children. Although the intravenous route of delivery and potential antigenicity of such receptor-decoys are of considerable concern in prolonged treatment of chronic HIV infection, these restrictions would not apply for similar drugs used for short-term treatment of acute arenavirus infection in humans. The development of receptor-based macromolecular anti-viral drugs targeting virus-host cell attachment represents therefore a promising approach in case of human pathogenic arenavirues. The identification of α-dystroglycan (α-DG) as the first cellular receptor for LFV (Cao et al., 1998) opens the possibility to design recombinant receptor-decoys to target the attachment of LFV to the host cell.

Upon receptor binding, arenavirus virions are internalized by uncoated vesicles and released into the cytoplasm by a pH-dependent membrane fusion step. The pH-dependent fusion between viral and cell membranes represents another potential drug target. While blocking of the fusion of virus with the cell

membrane has not yet been evaluated as an anti-viral strategy against arenaviruses, extensive experience with this type of approach has accumulated in the HIV field. After binding of HIV-1 GP120 to CD4 and the chemokine co-receptors, GP120 is thought to undergo conformational changes that expose gp41, the membrane-spanning portion of the HIV-1 envelope GP. Release of GP120 propels the previously sequestered fusion peptide of gp41 outward. While the fusion peptide inserts into the target cell membrane, exposed hydrophobic regions in the intertwined heptad-repeats (HR2) of gp41 interact with the gp41 HR1 coiled coils, forming a six-helix bundle. This "trimer of hairpins" configuration brings the viral and the cell membrane into close proximity and allows initiation of membrane fusion. In the past years a number of inhibitors have been developed that target this fusion process, among them enfuvirtide (T-20) and T1249, peptides that prevent the formation of the six-helix bundle intermediate. Despite important differences in membrane fusion between arenaviruses and HIV-1, like e.g. different cellular locations and distinct pH requirements, targeting of the fusion machinery present in arenavirus GPs appears as a viable option for the development of anti-viral drugs.

A.5 Small molecule inhibitors of protein interactions derived from combinatorial chemical libraries

Although LFVGP interacts with α-DG and possible yet unknown co-receptors likely by large molecular interfaces, a large number of studies on receptor-ligand binding indicate that in most cases, small clusters of amino acid residues mediate the energetically most important interactions. This leads us to expect that small molecules may be capable of blocking the virus-receptor interaction. Since no further structural information of the target proteins are currently available, we are using a combinatorial chemistry approach to search for synthetic antagonists of the binding of LFVGP to its receptor molecule(s). This strategy has recently been used successfully to identify several physiologically active inhibitors of novel biochemically not yet characterized interactions (Boger et al., 1998, Boger et al., 2000, Boger et al., 2001; Boger et al., 2003a/b, Silletti et al., 2001).

In Dr. Dale Boger's laboratory (Department of Chemistry, TSRI), solution-phase synthetic techniques have been developed to create combinatorial libraries of small molecules that can be used to screen for and identify molecules that promote protein–protein interactions (agonists) or inhibit protein–protein interactions (antagonists) (Fig. A5).

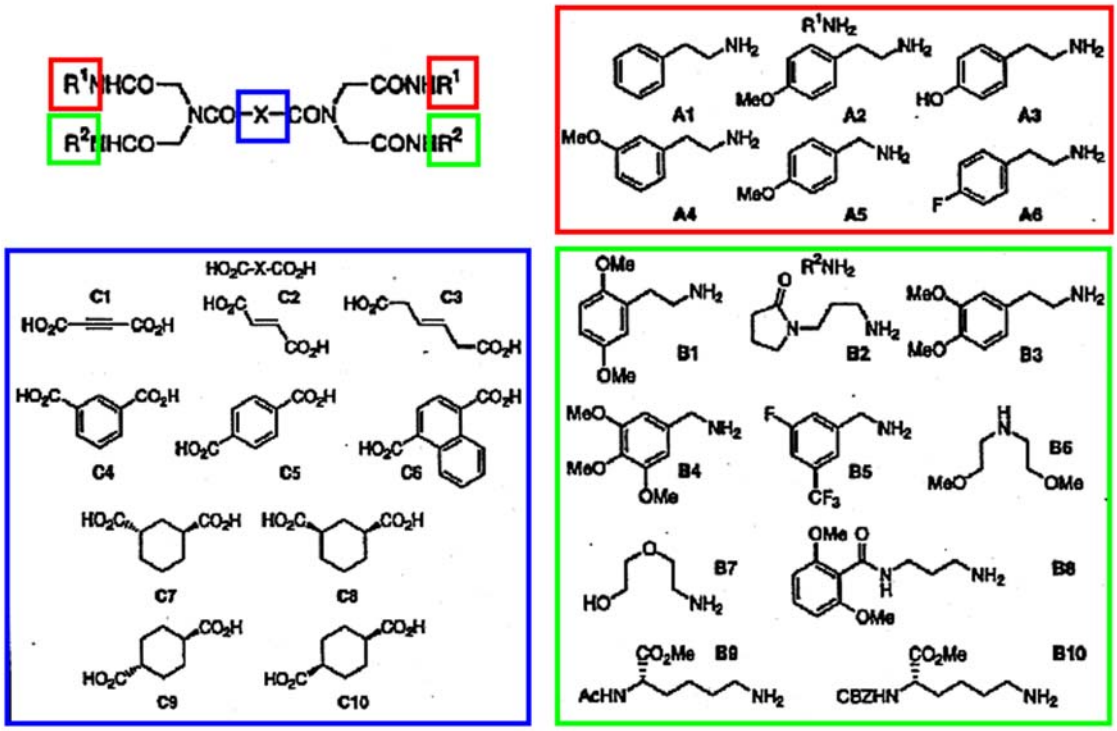

Figure A5: Example of a combinatorial chemical library used in our studies: I a first step, imididiacetic acid anhydride is coupled to the aromatic primary amines (A), resulting in 6 different products. Each of the products is then reacted with the compounds shown in B, resulting in a total of 60 compounds containing permutations of A and B substituents. The 60 compounds are then reacted with a mixture of 10 dicarbocyclic acids shown in C, resulting in 60 mixtures of 10 compounds with the general structure shown.

The multistep synthesis of such chemical libraries employing liquid–liquid and liquid–solid extractions to remove unreacted starting materials, reagents, and reagent byproducts provide the purified final products (>95% pure) irrespective of reaction efficiency.

Implementation is in formats for the parallel synthesis of individual pure compounds (1000 member libraries, individual compounds) and modest sized libraries composed of small mixtures (1,000–10,000 membered libraries, 10–50 compounds/mixture). This allows an approach to be adopted in a format compatible with our screening objective. Further, the compounds are not attached to solid supports making them immediately available for binding or functional assays. We presently have approximately 100,000 compounds in libraries that may be screened immediately and this collection grows at a pace of > 20,000 compounds/year.

Using this approach and Dr. Boger's libraries, erythropoieitin (EPO) mimetics (promote EPO receptor dimerization) (Goldberg et al., 2002), inhibitors of Myc/Max dimerization (Berg et al., 2002), and inhibitors of the binding of the protease MMP-2 to αvß3 integrin have been discovered (Silletti et al.,

2001; Boger et al., 2001). Based on the previous success with discovery and development of these small molecule inhibitors of protein–protein interactions, we plan to identify effective *in vitro* and *in vivo* inhibitors of viral infectivity from combinatorial chemical libraries

B. MATERIALS AND METHODS

B.1 Reagents and antibodies

Monoclonal antibodies (mAb) 33.1 and 83.6 (anti-LCMVGP2) have been described (Buchmeier et al., 1981; Weber and Buchmeier, 1988). The rabbit anti-GFP polyclonal Ab was from Chemicon. FITC-conjugated anti-rabbit IgG and phycoerythrin (PE)-conjugated anti-rat IgG were from Jackson Immuno-Research (West Grove, PA), and the HRP-conjugated anti-mouse IgG was from Pierce Chemical Co. (Rockford, IL). The Steady Glo® and Bright-Glo® luciferase assay systems were obtained from Promega (Madison WI). Heparan sulfate and heparin were purchased from Sigma.

B.2 Cell lines

African green monkey kidney (Vero-E6) cells were maintained in Dulbecco's modified Eagle medium (DMEM) (Gibco BRL, Grand Island, NY) containing 10% fetal calf serum (HyClone, Logan, UT). HEK293H cells were purchased from GIBCO BRL. For culturing as adherent cells, HEK293H were kept in complete serum-containing medium: DMEM, 10 % (vol/vol) FBS, supplemented with glutamine, and penicillin/streptomycin. For suspension cultures, cells were briefly trypsinized, washed twice in DMEM and re-suspended in the chemically defined serum-free medium 293 SFMII (GIBCO). The cell lines HeLa, A549 human lung carcinoma cells (ATCC CCL-185), and the packagic cell line GP293® (Invitrogen) were cultured in DMEM, 10 % (vol/vol) FBS, supplemented with glutamine, and penicillin/streptomycin. The glycosaminoglycan-deficient CHO cell line psgA-745 was obtained from ATCC. PsgA-745 cells and control wild-type CHO cells were kept in Ham's F12K medium supplemented with 10 % (vol/vol) FBS and 1.5 g/l sodium bicarbonate.

B.3 Virus strains, purification, and quantification

Origin, passage and characteristics of LCMV ARM53b and clone-13 have been described elsewhere (Dutko and Oldstone, 1983). Seed stocks of all viruses were prepared by growth in BHK-21 cells. Purified virus stocks were produced and virus titers determined as described. LFV Josiah was grown in Vero-E6 cells, polyethylene glycol-precipitated and γ-inactivated at the Center for Disease Control and Prevention in Atlanta GA.

Seed stocks of Pichinde were prepared by growth in BHK-21 cells. Purified virus stocks were produced and titers determined as described (Dutko et al., 1983). Amapari, Parana, Oliveros, Latino, Guanarito, Junin, Machupo, and LFV were grown in Vero-E6 cells in a BSL4 facility, polyethylene glycol-precipitated and γ-inactivated as described (Elliot et al., 1982). Inactivation was verified by a double blind passage of the inactivated viruses on Vero E6 cells followed by immunofluoresence staining for detection of viral antigen. Pichinde virus was inactivated by UV irradiation as described (Kunz et al., 2004). Inactivation was verified by plaque assay on Vero cells. The work with most of the infectious viruses was done at biosafety level 3 (BSL-3), with the exception of LFV, Guanarito, Junin, and Machupo viruses, which were handled in the BSL-4 laboratories at the Special Pathogens Branch, and Pichinde, which was handled at BSL-2 at the Scripps Research Institute.

B.4 Detection of LCMVGP in ELISA

Purified viruses were coated in triplicate wells in 96-well EIA/RIA high-bond microtiter plates (Corning) for 2 hours at 6 ºC and non-specific binding blocked with 1% (wt/vol) BSA/PBS. MAbs 83.6 (anti-LCMVGP2) was applied in 1: 100 dilution for 2 hours at 6 ºC and detected with peroxidase-conjugated anti-mouse IgG (1: 1000) in a color reaction using ABTS (2,2'azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)) substrate. OD (405) was measured with an ELISA reader. For the determination of specific binding, background binding to BSA was subtracted.

B.5 Immunoblotting

Proteins were denatured in hot (95 ºC) SDS-PAGE sample buffer: 2% (wt/vol) SDS, 50 mM Tris/HCl, pH 6.8, 100 mM DTT. Proteins were separated by gel electrophoresis and transferred to nitrocellulose using precast Novex® tris-glycine gels. After blocking in 5% (wt/vol) skim milk powder in PBS, membranes were incubated with the primary antibody mouse monoclonal antibody M2 anti-flag in a dilution of 1:1000 in 2% (wt/vol) skim milk powder, PBS for 12 hours at 6 ºC. After several washes in PBS, 0.1% (wt/vol) Tween-20 (PBST), the secondary antibody, goat anti-mouse IgG coupled to peroxidase was applied 1: 5000 in PBST for 1 hour at room temperature. Blots were developed by enhanced chemiluminescence (ECL) using Super Signal West Pico ECL Substrate (Pierce) and signals were recorded on autoradiographic film (Kodak, Rochester, N.Y.).

B.6 Molecular biology techniques

B.6.1 Polymerase chain reaction (PCR)

PCR profile

Segment#	duration (min)	temperature (ºC)	process
1	5	95	strand separation
2*	1	95	strand separation
3*	1	50	primer annealing
4*	1	72	extension
4	1	72	extension
5	for ever	4	cool down

*segments 2-4 were repeated 16 times

PCR reaction mixture

Reagent	final concentration	µl
PCR water	-	39.5
10 x pfu/taq buffer	1 x	5
dNTPs	200 µM/nucleotid	1
primer 1	500 nM	1.25
primer 2	500 nM	1.25
DNA template	500 ng	1
Pfu/Taq-polymerase	2.5 U	1

B.6.2 Phenol chloroform extraction

Phenol chloroform extraction was performed to remove contaminating proteins from DNA. 50 µL of phenol chloroform was added to the DNA solution. The mixture was vortexes on full speed for 1min and then spun on full speed (14 krpm) for 1 min. The upper phase was transferred to a new eppendorphtube and added 2.5 volumes of pure ethanol. The DNA precipitated at -70°C > 30 min (or overnight), then spun for 15 min at 14 krpm in the cold room. Supernatant was removed and pellet washed with 50 µL cold (-20°C) 75% ethanol. Ethanol was removed and the pellet air-dried. The pellet was dissolved in PCR water (30 µL) at 37°C. The amount and quality of the DNA fragment was checked by gel electrophoresis.

B.6.3 Agarose gel electrophoresis

Agarose gel electrophoresis can be used to distinguish and separate DNA fragments of different sizes. Small DNA fragments (<2.0 kilobases) are separated on gels with 1.0-2.0% agarose and larger DNA fragments (2.0-10.0 kilobases) are separated on gels with 0.6-1.0% agarose. The DNA molecules will run over the gel in the electrical field, migrating toward the anode. The speed of migration of the DNA molecules depends on their size; therefore the small molecules will migrate faster than those of the larger sizes because they more readily travel through the polymer matrix. To determine the size of the separated DNA molecules, they are compared to a DNA marker fragments of known size run on the same gel. Ethidium bromide is pre added to the gel and will interchelate between basepairs of the DNA double helix. This will emit light (fluorescence) exposed to UV light providing a means to detect the location of the DNA in the gel.

The agarose was dissolved in 100 ml 1x TAE buffer and heated to the boiling point in the microwave oven. 1 drop of ethidium bromide was added to the agarose and the solution was pored into a gel mold fitted with the appropriate comb. The gel was allowed to harden at room temperature. The comb was removed and the gel was transfered to an electrophoresis chamber and covered with 1x TAE buffer. DNA loading buffer was added to the DNA samples and they were loaded on the gel. A DNA marker was added in a free well. The gel was run at 120 V. The DNA was visualized under UV-light.

B.6.4 Gel extraction

The QIAGEN Gel Extraction Kit (QIAquick Gel Extraction Kit) was used for extraction of DNA from the gel.

The DNA fragment from the agarose gel was excised with a clean, sharp scalpel and weighed in a colorless tube. 3 volumes of Buffer QG were added to 1 volume of gel and this was incubated at 50°C for 10 min (until the gel slice had completely dissolved). 1 gel volume of isopropanol was added and mixed with the sample. A QIAquick spin column was placed in a provided 2 ml collection tube. To bind DNA, the sample was added to the QIAquick column, and centrifuge for 1 min. The flow-trough was discarded and the QIAquick column was placed back in the same collection tube. For washing, 0.75 ml of Buffer PE was added to QIAquick column and centrifuged for 1min. The flow-trough was discarded and the QIAquick column was centrifuged for an additional 1 min. The QIAquick column was placed into a clean 1.5 ml microcentrifuge tube. To elute DNA, 50 μL of Buffer EB was added to

the center of the QIAquick membrane, the column was standing for 1min, and then centrifuged for 1 min. For gel analyze of the extracted DNA, 1 volume of Loading Dye was added to 5 volumes of purified DNA. The solution was mixed by pipetting up and down and then loaded to the gel.

B.6.5 Treatment of DNA fragments with calf intestinal phosphatase (CIP)

The removal of terminal phosphate greatly reduces re-ligation e.g. of vector DNA and is essential for all vector DNA intended for use in ligation reactions. After completion of the restriction digest, calf intestinal phosphatase (CIP) is added to the reaction. CIP is compatible wit all four NEB buffers.
1 µl of CIP (Promega) was added to each preparative vector digest and the reaction was mixed thoroughly before it was incubated for 30 minutes at 37 ºC.

B.6.6 Ligation of DNA fragments with T4 DNA ligase

The DNA fragment and the vector DNA were separated in a preparative TAE gel. The DNA fragments were recovered from the gel using QIAEXII gel extraction. DNA fragments recovered by QIAEX were present in 20µl 1 mM Tris/HCl pH 8.0, which is ideal for subsequent ligations by T4 DNA ligase. For each ligation reaction, a control-reaction that contained only the vector DNA was included to assess the background of vector re-ligation.

Ligase reaction

Component	µl
DNA fragment (recovered by QUIAEX)	12
Vector DNA (recovered by QUIAEX)	5
10 x T4 DNA ligase buffer	2
T4 DNA ligase (NEB)	1

Reaction times: 2-16 h at 16ºC (water bath in cold-room)

B.6.7 Transformation of *E.coli*

The following protocol was used for the transformation of chemically competent strains of *E.coli* like e.g. DH5-α: The competent bacteria were thawed on wet ice. For transformation, 50 µL bacteria were needed, the rest of the competent bacteria were aliquoted in 50 µL aliquots that were snap frozen (i.e. frozen quickly in a cold bath prepared with ethanol and dry ice). The 50 µL bacteria were mixed with 5

μL of the ligation reaction and kept on ice for 30min and then put at 37°C for 30 seconds (heat shock). The reaction was put on ice for one minute and added 1 ml of LB medium without antibiotic. The reactions were shaken at 180 rpm for 40-60min at 37°C and then spun in microcentrifuge 14 krpm for 30 second. All LB medium was removed except of 50 μL of liquid so the bacteria could gently be re-suspended. The bacteria suspention were plated on Amp/LB plates and kept at 37°C for 16-24 h.

B.6.8 Plasmid DNA Purification Using the QIAprep Spin Miniprep Kit

The QIAGEN Miniprep Kit is used for plasmid DNA purification from overnight cultures of E.coli grown in LB medium. Circa 1 ml of each sample bacteria suspention was transfered to tubes, spun for 1min at 14 krpm. The supernatant was discarded and the pelleted bacterial cells were resuspended in 250 μL Buffer P1. 250 μL Buffer P2 was added to the tubes and mixed thoroughly by inverting the tubes gently 4-6 times. 350 μL Buffer N3 was added to the tubes and mixed immediately and thoroughly by inverting the tubes 4-6 times. The tubes were centrifuged for 10 min at 13 krpm in a table-top microcentrifuge. The supernatant from this last step was applied to the QIAprep spin column by decanting or pipetting. The columns were then centrifuged 60 s and the Flow-through were discarded.

For washing, 0.75 ml Buffer PE was added to the QIAprep spin columns before they were centrifuged for 60 s. The flow-through was discarded and the columns were centrifuge for an additional 1 min to remove residual wash buffer. The QIAprep columns were placed in a clean 1.5 ml microcentrifuge tubes. To elute DNA, 50 μL Buffer EB was added to the center of each QIAprep spin column. The columns were standing for 1 min before centrifugation for 1 min.

B.7 Generation of pseudotyped retroviral vectors

The packaging cell line GP2-293® from BD Bioscience stably expresses the Moloney mouse leukemia virus (MLV) derived gag and pol gene and allows packaging of any MMLV-based vector containing the appropriate packaging signal.

Co-transfection of a MLV-derived retroviral genomic plasmid pLZRS-Luc-gfp and the GP expression plasmid: GP2-293 cells were cultivated on plastic in T175 flasks to a confluency of 80%. The cells were split 1:2 into T175 flasks coated with poly-L-lysine. Medium for maintenance and

transfection: DMEM, 10% (v/v) FBS, Gln, P/S. Medium for virus production: DMEM, 10% (v/v) FBS, Gln, P/S, 20 mM Hepes. Transfection was carried out according to Sonderegger et al., Curr. Prot. Cell Biol. Unit 9.5. Briefly, 4 h prior to transfection, medium was removed and 40 ml of fresh medium was added per flask. Solution A was prepared (2 ml 0.25 M $CaCl_2$, 20 µg GP expression plasmid DNA (QiaGen Maxi-prep), 20 µg MLV genomic plasmid pLZRS-Luc-gfp DNA (QiaGen Maxi-prep)). Solution A was mixed into solution B (2 ml HBS) and incubated at room temperature for exactly 1 min. Transfection solution was added to cells and distributed evenly. Flasks were incubated at 37ºC, 5% CO_2 for 4-12 h. Transfection mix was removed and cells were washed twice with medium. Complete medium with 20 mM Hepes was added (25 ml/ flask) before the flasks were put back in the incubator. The pseudotypes were harvested after 24 and 48 hours, cleared by low speed centrifugation and frozen at –70ºC.

B.8 Infection of human cells with retroviral pseudotypes

Target cells: One day before the assay 2 x 10^4 HeLa cells were plated per well in flat bottom M96 plates (total volume medium 200 µl) in DMEM, 10% FBS, Gln, non-essential amino acids, Pen/Strep. The cells were circa 80% confluent at time of assay (after 16-24 hours).

Infection: Medium from target cells was removed, 100 µl pseudotypes was added to cells before they were incubated for 1 h at 37ºC, 5% CO_2. Mixture was removed and cells were washed twice with DMEM, 10 mM Hepes without FBS. 100 µl complete medium containing 10 mM Hepes per well were added and cells were incubated for 40-48 h at 37ºC, 5% CO_2. The results were read by Steady-Glo® luciferase assay.

B.9 Steady-Glo® luciferase assay

The Steady-Glo® luciferase assay is fully compatible with our medium (DMEM with 10 % FBS). The presence of phenol red reduced the signal intensity by circa 30%. We will avoid this problem in the future by using medium without phenol red.

The old medium was removed from the cells and replaced with 100 µl/well of fresh (37 ºC warm) medium. 100 µl assay (room-temperature) reagent/well were added to the plates. After 5 minutes, the cells were scratched off from the bottom of the plate and mix well with the solution (> 5 times up and down pipetting). Content of wells were transferred into a white M96 plate suitable for

chemiluminescence reader. Luminescence was measured in a 96-well plate luminometer (Bertold) using WinGlow software.

B.10 Screening of chemical libraries: inhibition of the infection with retroviral pseudotypes by compounds from combinatorial chemical libraries

Compound mixtures: The compound mixtures derived from combinatorial chemical libraries were dissolved in DMSO, in a concentration of 5 mM. We had 10 µl per compound mixture. The libraries were provided in 96 well plate formats. The pharmacologically active concentration in the primary screening assay, ideally 50 µM, corresponded to a 100-fold dilution of the stocks.

Pseudotypes: Retroviral pseudotypes containing the GPs of different arenaviruses (LFV, LCMV, Amapari, Guanarito, Junin, Machupo, and Vesicular stomatitis virus,) produced as described, were used in this assay.

Target cells: One day before the assay 2 x 10^4 HeLa cells were plated per well in flat bottom M96 plates (total volume medium 200 µl) in DMEM, 10% FBS, Gln, non-essential amino acids, Pen/Strep. The cells were circa 80% confluent at time of assay (after 16-24 hours).

Blocking pseudotypes with compounds: For the screening assay, exact replicas of the plates were done to avoid mix-up of samples. Empty wells of the compound plate were used for controls. 100 µl of pseudotypes in OPTIMEM, 2% FBS, 10 mM Hepes were added per well of a M96 plate and 1 µl of compound mixture were added per well and mixed briefly. For control wells DMSO only were added. Plates were incubated for one hour on ice.

Infection: Medium from target cells were removed and 80 µl of pseudotypes /compound mixtures were added to each well before plates were incubated for 1 h at 37ºC, 5% CO_2. After incubation, mixtures were removed and cells were washed once with DMEM, 20 mM Hepes without FBS. 100 µl complete medium containing 20 mM Hepes were added per well and plates were incubated for 40-48 h at 37ºC, 5% CO_2. The results were read by Steady-Glo® luciferase assay.

B.11 Target-specificity of compound mixtures

To assess the target-specificity of compound mixtures derived from combinatorial chemical libraries, compound mixtures were tested for their ability to block infection of target cells with arenavirus pseudotypes but not with pseudotypes containing the GP of the unrelated VSV. This was done by using the assay described in B9.

B.12 Determination of the dose-response characteristics of selected candidate compounds

Cells: Human lung epithel A549 in DMEM/10% FBS/Gln/PS
One day before the assay 2 x 10^4 cells were plated per well in flat bottom M96 plates (total volume medium 200 µl). At the time of assay, the cells were circa 80% confluent (after 16-24 hours).

Blocking of LFV-PS/VSV-PS with increasing concentrations of candidate compounds:

Concentration	**µl LFVPS**	**µl compound**	**dilution**
0	300	6	pure DMSO
1 µM	300	6	50 µM (1:100)
5 µM	300	5	500 µM (1: 10)
10 µM	300	6	500 µM (1:10)
20 µM	300	1.2	5 mM (stock)
50µM	300	3	5 mM (stock)
100 µM	300	6	5 mM (stock)

Infection in triplicates: Medium was removed from target cells, 90 µl of PS /compound mixtures were added to cells and plates were incubated for 1 h at 37ºC, 5% CO_2. After incubation, mixture was removed and cells were washed once with DMEM, 10 mM Hepes without FBS. 100 µl complete medium containing 10 mM Hepes was added per well and plates were incubated for 40-48 h at 37ºC, 5% CO_2. The results were read by Steady-Glo® luciferase assay.

B.13 Blocking of pseudotype infection with sulfated polysaccarides

Dextran sulfate and fucoidan stock solutions: 10 ml of solution of 10 mg/ml in 400 mM Hepes, pH 7.5 were prepared and filtered through 0.45 µM filter.

Target cells: One day before the assay, 2 x 10^4 Vero cells were plated per well in flat bottom M96 plates (total volume medium 200 µl) in DMEM, 10% FBS, Gln, non-essential amino acids, Pen/Strep. At the time of assay, the cells were circa 80% confluent (after 16-24 hours).

Retroviral pseudotypes: LFV, Guanarito, Junin, Machupo, VSV were done in triplicate samples, added 100 µl of pseudotypes per well.

Blocking of pseudotypes with dextran sulfate or fucoidan: Compounds were added to the pseudotypes according as follows:

HS/heparin (µg/ml)	µl pseudotypes	µl PCM	µl HS/heparin stock
0	360	40	0
0.2	360	32	8 (10 µg/ml stock)
1	360	0	40 (10 µg/ml stock)
5	360	38	2 (1 mg/ml stock)
25	360	30	10 (1 mg/ml stock)
100	360	0	40 (1 mg/ml stock)

Infection: Medium from target cells were removed and 100 µl of the pseudotype mix were added to each well and incubated for 1 h at 37ºC, 5% CO_2. After incubation, mixture was removed and cells were washed once with PCM, 20 mM Hepes. 200 µl PCM containing 20 mM Hepes were added per well and incubated for 40-48 h at 37ºC, 5% CO_2. The results were read by Steady-Glo® luciferase assay.

B.14 Intracellular FACS staining for LCMV-NP using mAb 113

Preparation of a single cell suspension: The cells cultivated in 6 well plates were washed twice with PBS. 1 ml/well of Trypsin-EDTA were added to the cells but not removed completely, leaving some liquid left in each well. The plates were incubated at 37ºC for 5 minutes, the cells were detach by slapping against the plate and re-suspend in 1ml/well 1% FBS/PBS. The cell-suspension were transferred to 5 ml polystyrene tube and kept on ice. The tubes were spun 1600 rpm/4min/4^{o}C and supernatant was removed. Cells were re-suspend in 2 ml of 1% FBS/PBS. The tubes were again spun 1600 rpm/4min/4^{o}C and supernatant was removed. The cells were re-suspend in 250 µl of 1% FBS/PBS and kept on ice. 200 µl of each cell-suspensions were transferred into a M96 plate with conical wells.

Fixation and permeabilization: The cells were spun down 1600 rpm/4min/4^{o}C and supernatant were removed. Cells were resuspended in 100 µl/well of 4% (w/v) PFA/PBS and kept at RT for 10 min. The cells were washed twice with 1% (v/v) FBS/0.1% (w/v) saponin/PBS.

Wash procedure: The cells were spun down 1600 rpm/4min/4^{o}C and re-suspend in 200 µl/well of 1% (v/v) FBS/0.1% (w/v) saponin/PBS.

Intracellular staining with mAb 113 anti LCMVNP: mAb 113 was diluted 1: 100 in 1% (v/v) FBS/0.1% (w/v) saponin/PBS. Cells were resuspended in 100 µl/well of Ab solution and incubated for 45 minutes on ice in the dark. Cells were wash twice with 1% (v/v) FBS/0.1% (w/v) saponin/PBS.

Detection with FITC-conjugated 2nd Ab: 2nd Ab anti-mouse IgG F(ab)$_2$-FITC was diluted 1: 100 in 1% (v/v) FBS/0.1% (w/v) saponin/PBS. Cells were re-suspend in 100 µl/well of 2nd Ab and incubated for 45 minutes on ice in the dark. After incubation, the cells were washed once with 1% (v/v) FBS/0.1% (w/v) saponin/PBS and twice with 1% FBS/PBS. Cells were re-suspend in 200 µl/well PBS and kept in the dark.

B.15 Detection of LCMV-NP by immunofluorescence staining

Cells: 10^4 Vero E6 cells were plated per well of a 8 well LabTek tissue chamber slides (Nunc) and cultured over night.

Infection with LCMV: LCMV was added at MOI of 0, 0.01, 0.1, and 1 in an inoculum of 250 µl medium for 45 minutes at 37ºC, 5% CO_2. After infection, cells were washed and 0.5 ml fresh medium per well were added.

Fixation of cells: The culture medium was removed and 250 µl of 2% formaldehyde/0.1% glutaraldehyde in PBS (room temperature) was added to each well for fixation of the cells at 37^{o}C for 15 minutes in the dark. The formaldehyde fixative was aspirated and cells were washed twice with PBS. PBS was removed after the second wash step and 500 µl PBS/1% (v/v) FCS were added and cells incubated for 15 min at room temperature.

Immunofluorescence staining

Permeabilization of cells: After the blocking step (#2), 250 µl PBS/1% (v/v) FCS/0.1% (w/v) saponin was added and cells incubated for 15 min at room temperature.

Incubation with primary antibody: Primary antibody: mAb 113 anti-LCMVNP 1: 200 in PBS/1% (v/v) FCS/0.1% (w/v) saponin (250 µl/well) was added and cells incubated for 1h at room temperature.

Incubation with secondary antibody: The secondary antibody (goat anti mouse IgG FITC labeled) was diluted 1:100 in PBS/1% (v/v) FCS/0.1% (w/v) saponin. Primary antibody solution was removed and cells were washed 2 times with PBS. 200 µl/well secondary antibody in PBS/1% (v/v) FCS were added and cells were incubated for 45 min at room temperature, protect from light. Secondary antibody solution was removed and cells were washed 3 times with PBS.

Evaluation: Fluorescence microscope with the 5x objective was used for evaluation. All NP+ cells were counted and cell clusters were scored as one infection event.

C. RESULTS

C.1 Discovery of novel small molecule inhibitors of Lassa fever virus (LFV) infection from combinatorial chemical libraries:

C.1.1 Production and characterization of retroviral pseudotypes

Since LFV is a BSL4 pathogen, studies with live virus are restricted to BSL4 laboratories. To discover small molecule drugs that can block the infection of human cells with LFV, our laboratory has generated retroviral vectors that contain the glycoprotein (GP) of LFV in their envelope. The principle behind this is the fact that enveloped viruses can incorporate heterologous viral GPs into their lipid membranes during budding. These so-called “pseudotypes” acquire the receptor specificity of the virus from which the heterologous GP was derived.

Using the strategy outlined in Fig. C1A, we inserted the GPs of LFV strain Josiah and the LCMV isolates ARM53b (ARM) and clone-13 (cl-13) into virions of recombinant Moloney leukemia virus (MLV), which contain a luciferase and a green fluorescent protein (GFP) reporter gene. In addition, we generated pseudotypes containing the GPs of the New World arenavirus Amapari (AMA) and of vesicular stomatitis virus (VSV), which do not use α-DG as a receptor (Cao et al., 1998; Spiropoulou et al., 2002).

The packaging cell line GP2-293-Luc® (BD Biosciences) stable expresses MLV gag and pol. The cells were transfected with a plasmid expressing a packable MLV genome pLZRS-Luc-GFP and an expression plasmid for LFVGP. pLZRS-Luc-GFP was developed by the laboratory of Dr. Gary Nabel (Yang et al., 1998) and contains a luciferase transgene and a green fluorescence protein (GFP) reporter encoded in a bicistronic mRNA. This guarantees a defined ratio of luciferase and GFP being expressed in cells transduced with the recombinant retrovirus. For transfection, I used calcium phosphate, a technique that results in high efficiencies of transfection. In this method, the formation of the DNA-containing calcium phosphate particles is initiated under defined chemical conditions, in the absence of cells or serum. The particle size is the most critical parameter regarding efficiency of transfection, that is uptake of DNA-containing calcium phosphate particles by the cell. The main determinations of particle size are calcium and phosphate concentration, the concentration of the DNA, size of the DNA fragments involved, pH, temperature and time of incubation. After initial formation of the DNA-containing calcium phosphate particles, the precipitate is added to the cells. During the incubation of

the precipitate with the cells, the formation of DNA-containing calcium phosphate particles continues and preexisting particles grow in size. The particles adhere to the cells and are taken up by endocytosis. After a few hours of exposure, the medium is changed and the cells start to express the recombinant pseudotypes. The pseudotypes are released from the cells and accumulated in the cell supernatant. After 48 hours, supernatants were harvested, centrifuged at low speed to remove cell debris, aliquoted and frozen at –80 ºC.

The titers of the pseudotypes were determined by infection of Vero cell monolayers, using the GFP reporter. Briefly, serial dilution of pseudotypes were made and put on monolayers of Vero cells in 96 well plates. After one hour of incubation, cells were washed and fresh medium added. After 48 hours, the cells were fixed with 2% formaldehyde and immunostaining for GFP performed using a specific antibody to GFP. Clusters of green cells were counted and scored as infectious uints (iu). Representative titers for arenavirus pseudotypes are 1-4 x 10^5 infectious particles per ml (iu/ml) and of VSV pseudotypes (VSV-PS) 5-10 x 10^5 iu/ml. Infection of permissive cells was reliably detected by luciferase assay (Fig. C1B)

An initial characterization of the pseudotypes has already been done in the lab before I arrived. Although not part of my own work, the main results are displayed Fig. C1 (C and D). As can be seen, the arenavirus pseudotypes were specifically recognized by the monoclonal antibody (mAb) 83.6, which binds to a highly conserved epitope in arenavirus GP2 (Weber and Buchmeier, 1988) (Fig. C1C). This proofs that the GP of the heterologous virus was efficiently incorporated into the MLV particles during budding.

Infection of cells by pseudotypes containing the GPs of LFV (LFV-PS), LCMV (LCMV-PS), and AMA (AMA-PS) was specifically blocked by the corresponding inactivated viruses, but not by inactivated Parana virus (PAR), an unrelated arenavirus, which belongs to the Clade A New World arenaviruses (Fig. C1D). Inactivated LFV, AMA, and PAR were provided by the Special Pathogens Branch at the Center of Disease Control in Atlanta, GA as described (Spiropoulou et al., 2002).

These data indicate that the retroviral pseudotypes adopt the receptor binding specificities of the viruses from which their GPs were derived.

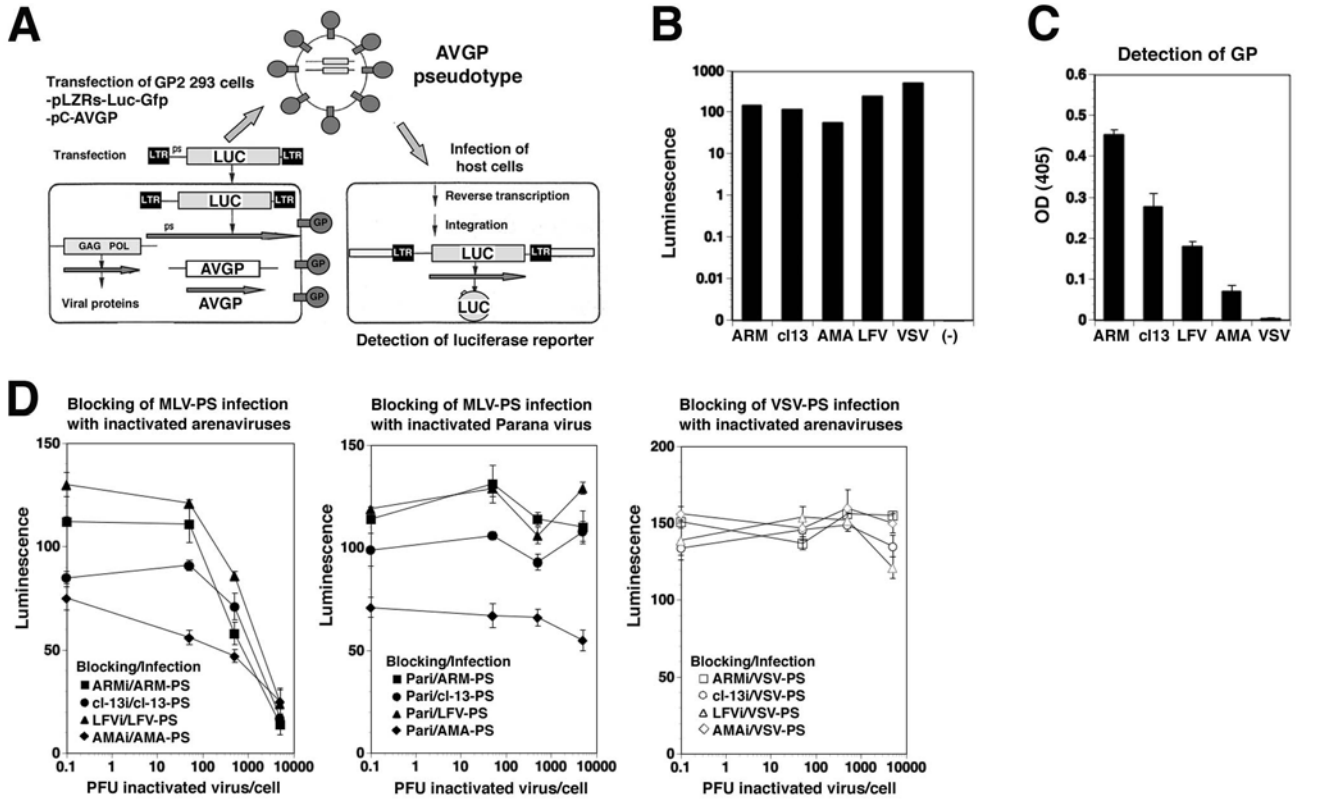

Figure C1: Recombinant retroviral vectors pseudotyped with arenavirus GPs. (A) Production of pseudotyped retroviruses: The packaging cell line GP2-293® (BD Biosciences) stable transfected with MLV gag and pol, is co-transfected with a plasmid containing the packable MLV genome pLZRS-Luc-Gfp (Yang et al., 1998), which contains a luciferase and a GFP reporter, and an expression plasmid for the heterologous GP (pC-AVGP). Retroviral pseudotypes are released into the cell supernatant. Infection of target cells results in the integration of the retroviral genome into the host cell DNA and can be quantified by detection of the luciferase or GFP reporter. (B) Infection of cells with retroviral pseudotypes: Supernatants containing pseudotypes were used to infect monolayers of Vero cells in 96 well plates. After 48 h, infection was quantified using the Steady-Glo® luciferase reporter gene assay (Promega) in a Berthold 96 well-plate luminometer. Luminescence is expressed as fold-increase over uninfected controls (n = 3 $\pm$ SD). (C) Binding of mAb 83.6 to pseudotypes: Equal amounts of concentrated pseudotypes containing the GPs of LCMV ARM53b (ARM), cl-13, LFV, AMA, and VSV were immobilized in microtiter plates and probed with mAb 83.6 anti-LCMVGP2. Primary antibodies were detected with POD- conjugated anti-mouse IgG in a color reaction using ABTS substrate. OD (405) was measured using an ELISA reader (n = 3 $\pm$ SD). (D) Blocking of arenavirus pseudotype infection by inactivated viruses: HEK293 cells cultured in 96-well plates were blocked with inactivated LCMV (ARMi, cl-13i), LFV (LFVi), AMA (AMAi), or PAR (Pari) at the indicated ratios of inactivated virus particles per cell. After blocking for two hours at 4 ºC, cells were infected with the indicated pseudotypes at a multiplicity of infection (MOI) = 1. Infection was assessed by luciferase assay as in (B) (n = 3, $\pm$ SD).

C.1.2 Screening of combinatorial chemical libraries

The Combinatorial chemical libraries were given to us by the laboratory of Dr. Dale L. Boger (Department of Chemistry, Scripps). The libraries were provided in 96 well plates containing either individual compounds or mixtures of 4-10 compounds in a concentration of 5 mM in DMSO. The compounds/compound mixtures were screened for their ability to block infection of target cells by retroviral pseudotypes that contain LFVGP in their envelope and a luciferase reporter gene.

For each library, two independent screenings were performed as shown in Fig C2 and only 90 compounds that showed >50% inhibition of infection in both independent screens were considered candidates to be followed up. During my time in the laboratory, I have screened 13 complete libraries with total > 10, 000 iminodiacetic acid-based and pyrrolidine-based peptidomimetic compounds. Duplicate screening resulted in the identification of circa 4-5% compounds/mixtures that showed reproducible reduced LFV-PS infection by > 50%.

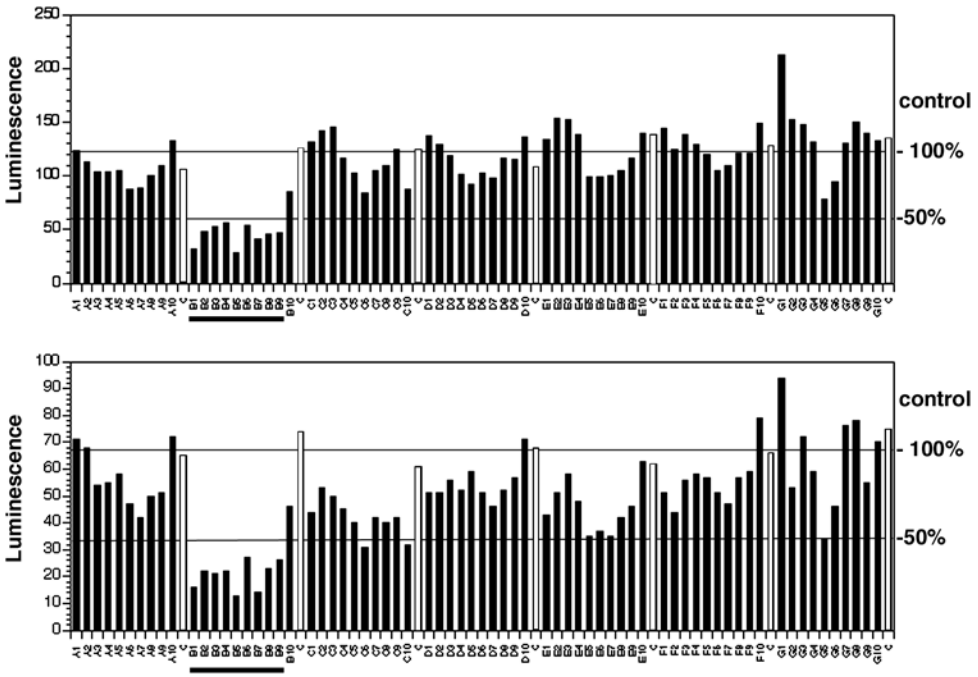

Figure C2: Example of the high-throughput screening of a combinatorial chemical library for inhibitors of LFV-PS infection. LFV-PS were pre-incubated with compound mixtures (concentration = 50 µM for individual compounds and 5 µM for individual compounds in mixtures of 4-10) for 45 minutes and then added to monolayers of HeLa cells in 96 well-plates. After one hour, the inoculum was removed, cells washed and incubated for 48 h. Infection was quantified using the Steady-Glo® high sensitivity luciferase reporter gene assay (Promega) in a Berthold® 96 well-plate luminometer. Data shown are two completely independent experiments screening the same library (#19). Luminescence is expressed as fold-increase over background.

A1-10, B1-10, C1-10, D1-10, E1-10, F1-10, and G1-10 (black bars) represent mixtures of seven different compounds each. The samples labeled C (white bars) correspond to solvent only controls. 100% and 50% of mean control values are indicated as horizontal lines. The mixtures B1-B9, which result in >50% inhibition of LFV-PS infection in both screens (underlined), showed the best effect for inhibition.

C.1.3 Determination of the target specificities of candidate compounds

Next, we wanted to select for compounds that specifically block LFVGP-mediated attachment and entry but do not influence subsequent steps of our retroviral pseudotype-based assay. To this end, we compared the effect of our 125 remaining candidate compounds/mixtures on infection with LFV-PS with infection with pseudotypes containing the GP of vesicular stomatitis virus (VSV-PS). VSVGP is structurally unrelated to LFVGP and binds to a different receptor. Our counter-selection is based on the fact that compounds, which specifically block LFVGP-mediated infection, should not interfere with VSV-PS infection while compounds that affect subsequent steps of the assay would reduce reporter gene expression in both, LFV-PS and VSV-PS. Determination of target specificity (Fig. C3) revealed that most compounds specifically reduced infection with LFV-PS but not VSV-PS.

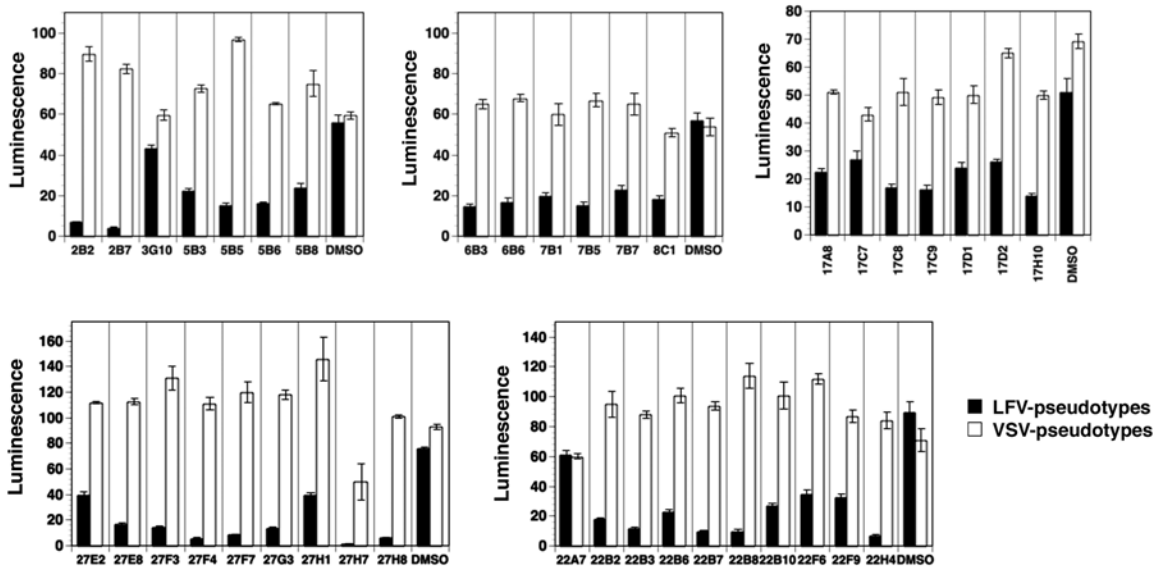

Figure C3: Determination of target specificity of representative candidate compounds/mixtures that showed significant blocking of LFV-PS infection in the screening assay: LFV-PS and VSV-PS were pre-incubated with compound mixtures as in Fig. C2 and then added to monolayers of HeLa cells in 96 well-plates. After one hour, the inoculum was removed, cells washed twice with DMEM and incubated with fresh medium for 48 h. Infection was quantified using the Steady-Glo® high sensitivity luciferase reporter gene. Data shown are triplicates (+/- SD). The samples labeled DMSO correspond to solvent controls.

C.1.4 Validation of candidate compounds in different human and primate cells

My own screens together with those done by other people of > 50, 000 compounds resulted in the identification of 157 (4.5%) compounds/mixtures that showed reproducible reduced LFV-PS infection by > 50%. When tested for cytotoxicity using 7-amino-actinomycin, a fluorescent dye that stains dead or damaged cells, most compounds/mixtures (125/147) derived from iminodiacetic acid-based and pyrrolidine-based peptidomimetic libraries showed no or only mild toxicity, while heterocyclic compounds (10/10) turned out to be toxic. Determination of target specificity (see Fig. C3 for representative examples) revealed that 32 single compounds and 53 compound mixtures out of a total of 125 specifically reduced infection with LFV-PS but not VSV-PS.

A selection of 17 single candidate compounds was then tested for their ability to specifically block LFV-PS infection in a series of susceptible human and primate cell lines including HeLa, A549, and VeroE6 cells. The majority of candidate compounds showed consistent blocking of LFV-PS infection in all cell lines tested (Fig. C4), indicating that the anti-viral activity was independent of the specific target.

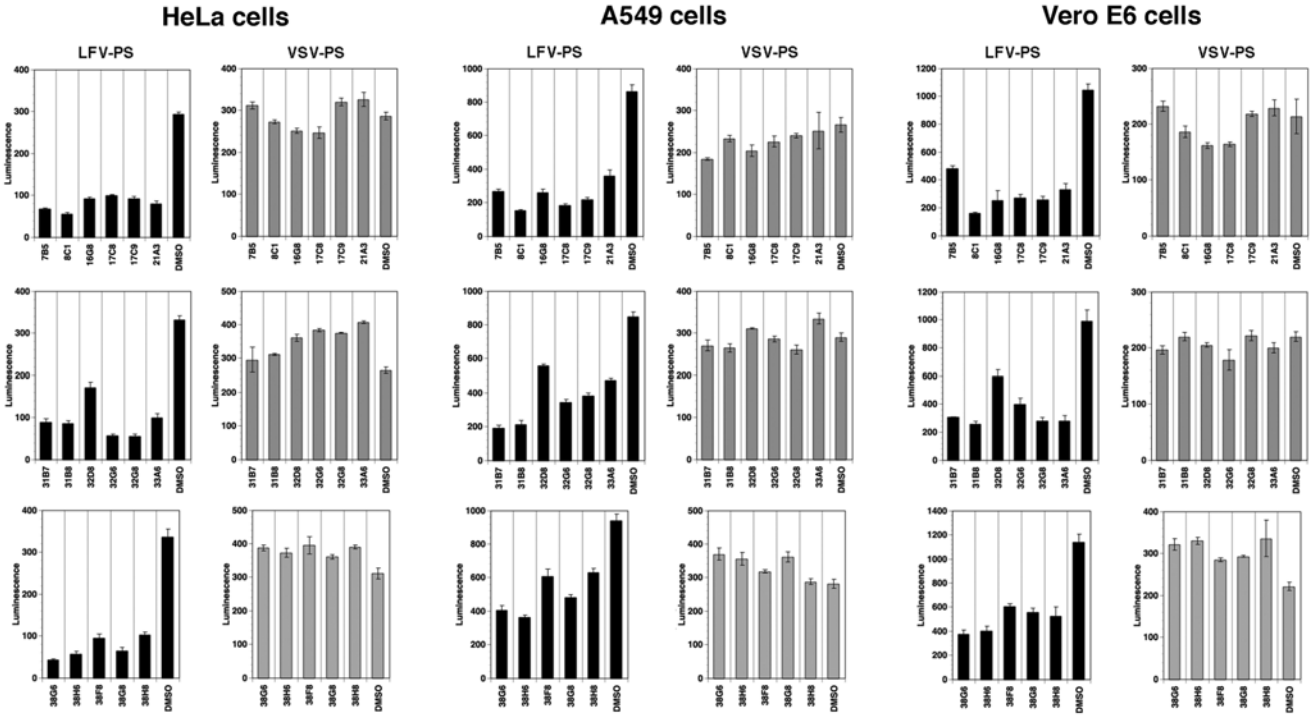

Figure C4: Activity of selected candidate compounds in different cell lines: Selected candidate compounds were tested for their ability to specifically inhibit LFV-PS infection in the susceptible human cell lines HeLa and A549 and the primate cell line VeroE6. LFV-PS and VSV-PS (negative control) were pre-incubated with the compounds at 50 µM and the added to cell monolayers. Infection was determined by luciferase assay. As can be

seen, of the 17 compounds tested in the different cell lines only eight showed highly consistent activity in all cell types: 7B5, 8C1, 16G8, 17C8, 17C9, 21A3, 31B7 and 31B8. The other ones, in particular compounds derived from library #38 showed different activities in different cell lines. While all compounds were highly active in HeLa cells, the cell type used for screening, some of the compounds showed only weak inhibition in A549 cells. The reasons for these discrepancies are not fully understood, but a possible explanation will be given later on in the discussion of my work.

C.1.5 Dose-response curves of candidate compounds

A next important step was the determination of the dose-response curves of the individual compounds. For this I focused on the eight candidate compounds, 7B5, 8C1, 16G8, 17C8, 17C9, 21A3, 31B7 and 31B8, that showed the best results when tested on the different cell lines (Fig. C4). LFV-PS were pre-incubated with increasing concentrations of compound mixtures for 1 hour and then added to monolayers of human lung epithelial A549 cells in 96 well-plates. After one hour, the inoculum was removed, cells washed and incubated for 48 h. Infection was quantified using the Steady-Glo® high sensitivity luciferase reporter gene assay (Promega) in a Berthold® 96 well-plate luminometer. Each concentration of the different compounds was done in triplicates. The dose response curves are given in Fig. C5.

C.1.6 Determination of the activity of candidate compounds against other human pathogenic arenaviruses

Apart from LFV, the arenavirus family contains other important human pathogens, such as the South American hemorrhagic fever viruses Guanarito, Junin, and Machupo. We therefore wanted to see if our candidate compounds that were active against LFV were also able to inhibit the infection of human cells with these viruses. To study the anti-viral activity of our compounds against the South American hemorrhagic fever viruses under BSL2 conditions, we used retroviral vectors that contain the glycoproteins (GPs) of Guanarito, Junin, Machupo, and the non-pathogenic New World arenavirus (NWAV) Amapari following the strategy used for the production of LFV pseudotypes (Fig. C1). The results of the original characterization done by Ms. Jillian Rojek in the lab are shown in Fig. C6. Briefly, infection of susceptible cells was detected using the luciferase reporter present in the retroviral genome (Fig. C6A). Infection of cells by pseudotypes was specifically blocked by the corresponding inactivated viruses, but not by inactivated LFV (Fig. C6B).

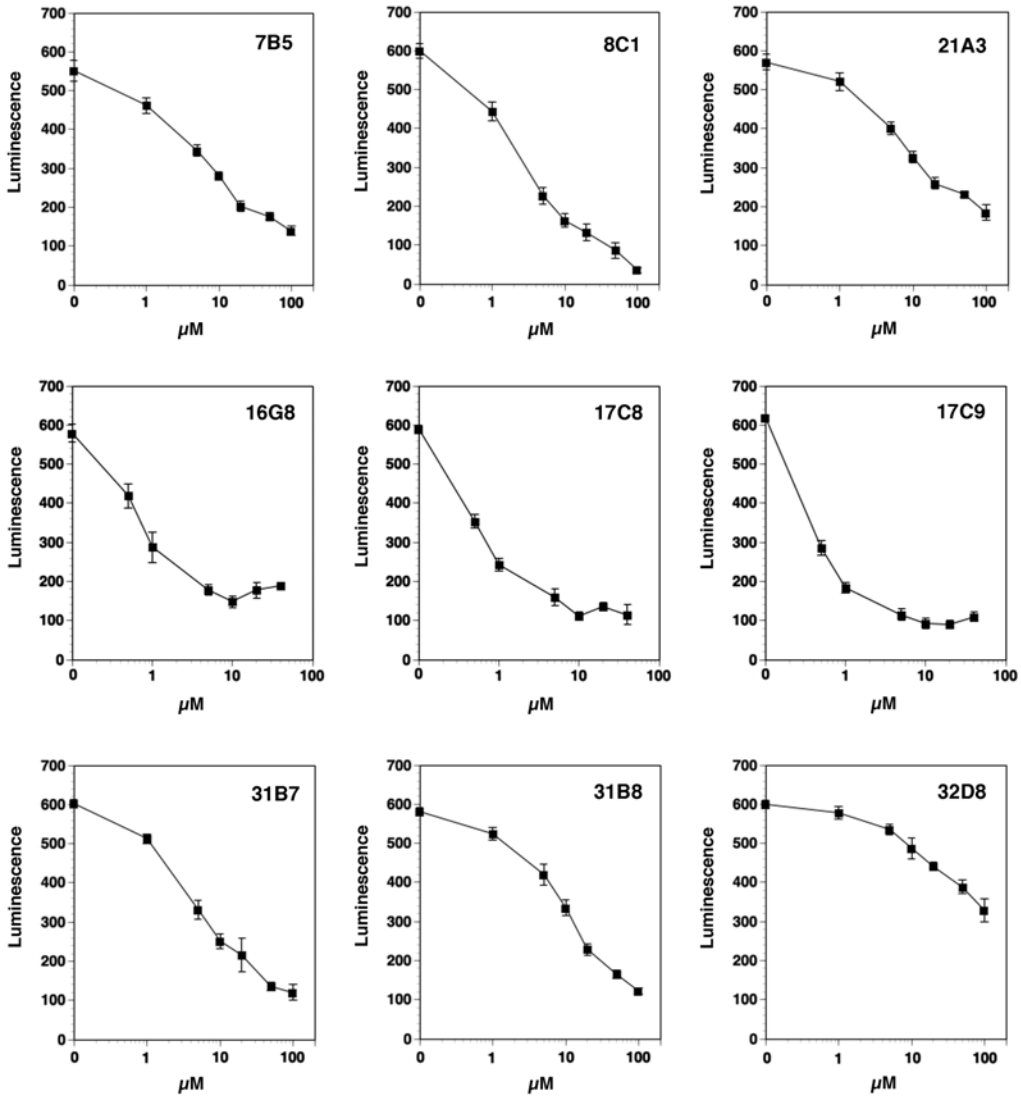

Figure C5: Determination of the dose-response curves for 9 selected candidate drugs. LFV-PS were pre-incubated with increasing concentrations of compound mixtures for 1 hour and then added to monolayers of human lung epithelial A549 cells in 96 well-plates. After one hour, the inoculum was removed, cells washed and incubated for 48 h. Infection was quantified using the Steady-Glo® high sensitivity luciferase reporter gene assay (Promega) in a Berthold® 96 well-plate luminometer.

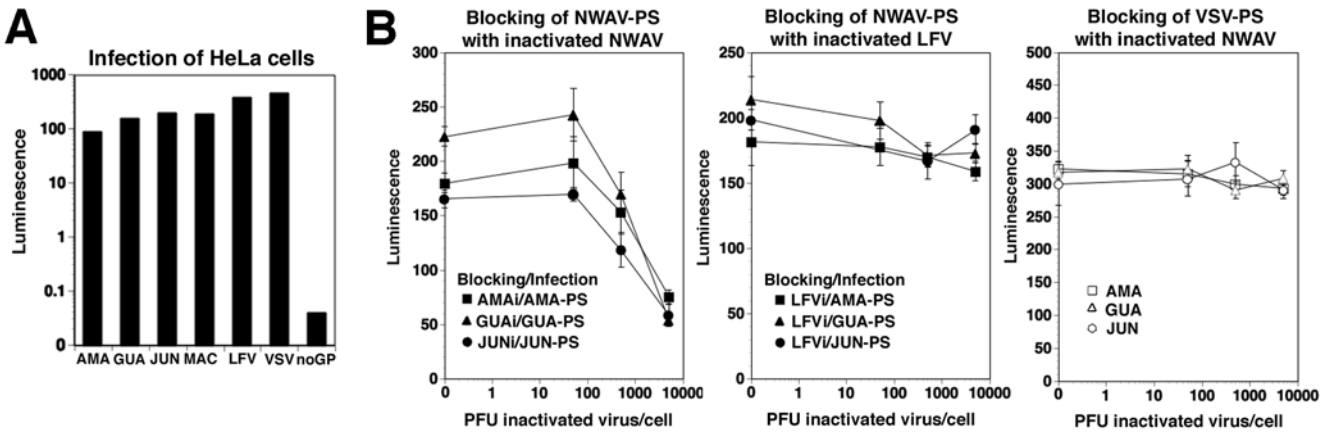

Figure C6: Recombinant retroviral vectors pseudotyped with NWAV GPs. (A) Infection of cells with retroviral pseudotypes: Undiluted supernatants of pseudotypes were added to monolayers of HeLa cells in 96 well-plates and infection quantified after 48 hours using luciferase reporter gene assay. Luminescence is expressed as fold-increase over noninfected control cells (n = 3 $\pm$ SD). (B) Blocking of New World arenavirus GP pseudotypes (NWAV-PS) infection by inactivated viruses: HEK293 cells cultured in 96-well plates were blocked with γ-inactivated Amapari (AMAi), Junin (JUNi), Guanarito (GUAi) or LFV (LFVi) at the indicated ratios of inactivated virus particles/cell. After incubation for two hours at 4 ºC, cells were infected with either NWAV pseudotypes or pseudotypes containing VSVGP (VSV-PS) at a multiplicity of infection (MOI) = 1. Infection was assessed after 48 h by luciferase assay (n = 3, $\pm$ SD).

The 17 selected candidate compounds were tested for their ability to block the infection of human cells (A549) with retroviral pseudotypes containing the GPs of Guanarito, Junin, and Machupo. To ensure the specificity of the observed reduction in infection by the candidate drugs, we used VSV pseudotypes as a negative control. Interestingly, many compounds that showed activity against LFV pseudotype infection were also active against the South American HF viruses (Fig. C7).

Many of the candidate compounds that showed specific activity against LFV-PS showed significant activity against pseudotypes of Guanarito, Junin and Machupo. The responses to the compounds in the different NWAV-PS are very similar. In particular the compounds 16G8, 17C8 and 17C9 showed nearly full neutralizing potential against these pseudotypes. The NWAV-PS are all over a bit more sensitive for neutralizing than the LFV-PS, otherwise the NWAV-PS respond more or less consistent to how the LFV-PS respond to the compounds.

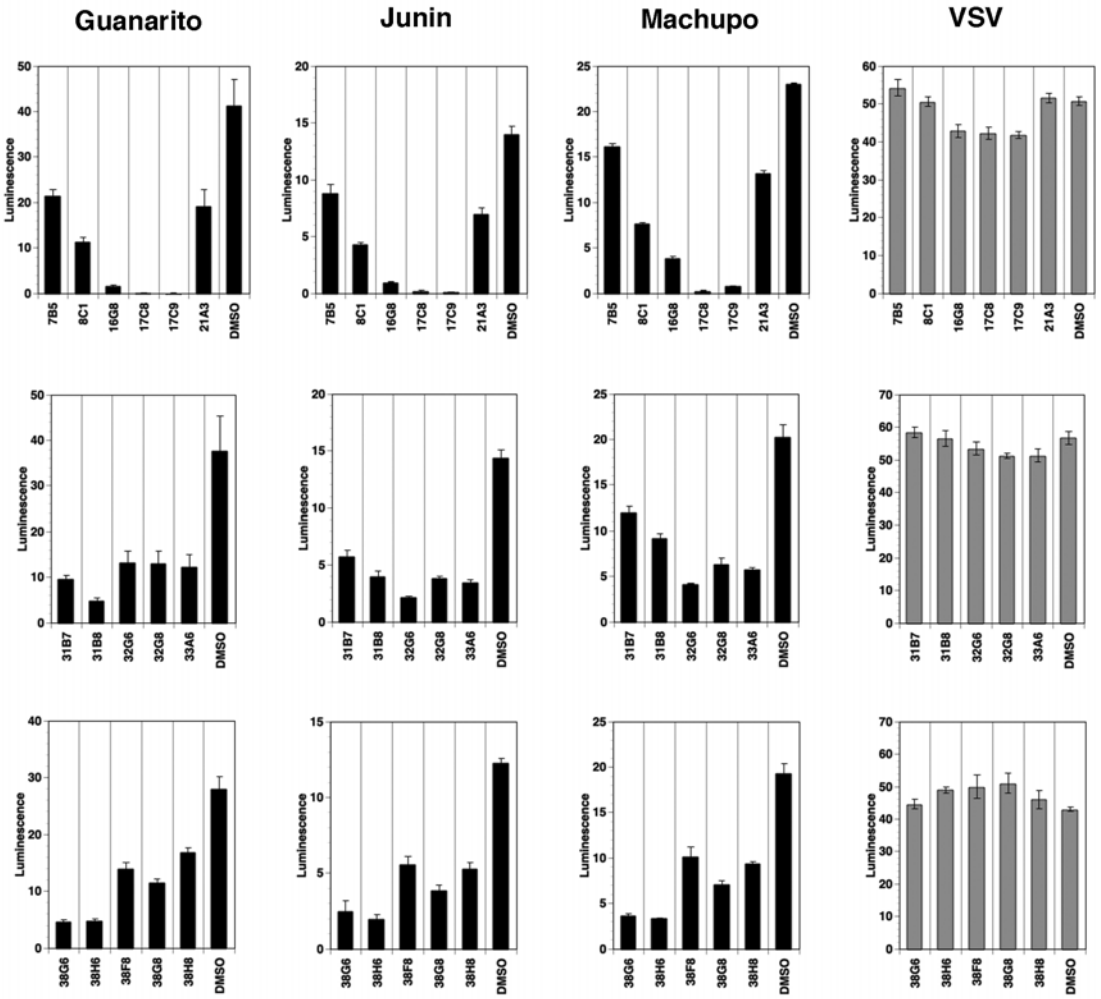

Figure C7: Activity of candidate compounds against retroviral pseudotypes of Guanarito, Junin, and Machupo. Candidate compounds were tested for their ability to specifically inhibit infection in the susceptible human cell line A549. Retroviral pseudotypes of Guanarito, Junin, and Machupo and vesicular stomatitis virus (VSV) (negative control) were pre-incubated with the compounds (50 µM) for one hour and then added to cell monolayers. Infection was determined by luciferase assay after 48 hours (n = 3 $\pm$ SD).

C.2 Discovery of novel small molecule inhibitors of Lassa fever virus (LFV) infection from natural product libraries:

During the past twenty years, several research groups have tested synthetic and natural compounds for activity against arenaviruses and found some anti-viral activity. Trifluroperazine (TFP) and chlorpromazine (CPZ), two pharmacologically active phenothiazine derivates, were found to be active on the replication of the arenaviruses Junin, Tacaribe virus and Pichinde virus (Candurra et al., 1996). Two myristic acid analogs (DL-2-hydroxymyristic acid (2OHM) and 13-oxamyristic acid (13OM) showed to have effects on Junin virus replication (Cordo et al., 1999*)*. Ribavirin triphosphate (RTP) can interact with various viral RNA polymerases and inhibit virus replication (Parker, 2004). Synthetic brassinosteriod ((22S,23S)-3ß-bromo-5α,22,23-trihydroxystimastan-6-one) has an antiviral mode of action against Junin virus replication by preventing the synthesis of full length antigenomic RNA (Castilla et al., 2005).

Since some of the compounds that show anti-viral activity like, hydroxymyristic acid, ribavirin triphosphate, and brassinosteriods are natural products or derivatives thereof, we decided to perform a broad screen of natural products for anti-viral activity. For this purpose, we used a natural product library of 2, 000 defined and characterized natural compounds. The compounds were provided by the Department of Chemistry at Scripps. The library was set up as 200 mixtures of 10 compounds each in a concentration of 500 µM per compound in DMSO. In contrast to the combinatorial chemical libraries used in our small molecule screens described above, the individual compounds present in the mixtures of this library have no structural relatedness. The natural compounds are tested for inhibition for cell-receptor binding and/or entry.

C.2.1 Screening of natural product libraries

Screening of natural product libraries was done in the same way as for the combinatorial chemical libraries, but in addition to LFV-PS we tested the compounds for effect on Guanarito, Junin, and Machupo pseudotypes. Initial screening of the three natural product libraries N1-N3 resulted in the identification of a number of compound mixtures (with 10 compounds each) that significantly reduced infection of human cells with either LFV or Machupo virus pseudotypes. To select for compounds that block specific one of the respective psudotypes-mediated attachment and entry but do not influence subsequent steps of our retroviral pseudotype-based assay, we compared the effect of our candidates

on infection with LFV-PS with infection with pseudotypes containing the GP of vesicular stomatitis virus (VSV-PS).

When we tested the active mixtures of natural compounds for target specificity using infection with VSV pseudotypes as a control, most mixtures showed activity also against VSV-PS (Fig. C8). Out of the 23 active compound mixtures tested, the mixtures N1B3, N1E5, N1G5, N1D6, N2A1, N2G8, N3D2 and N3F2 showed specific activity against LFV or Machupo, but not VSV.

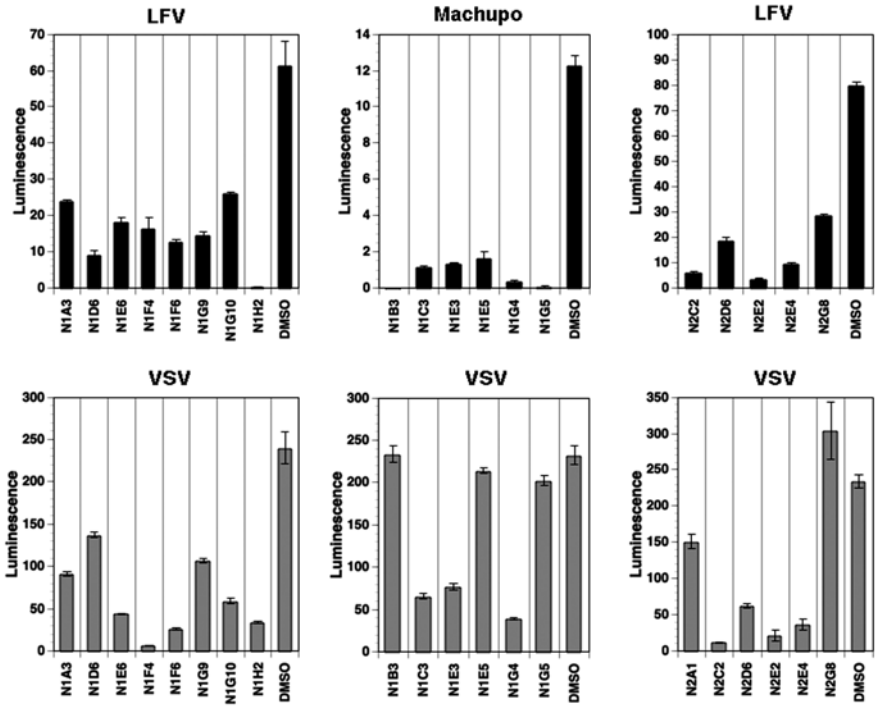

Figure C8: Target specificity of candidate mixtures derived from natural product libraries. Candidate mixtures from natural product libraries (10 compounds, 50 µM each) were pre-incubated with LFV-PS, Machupo pseudotypes and VSV-PS for one hour on ice and then added to HeLa cells. Infection was assessed after 48 h by luciferase assay (n = 3 $\pm$ SD).

C.2.2 Deconvolution of compound mixtures

To identify the active compounds in each mixture, we re-screened the individual compounds present for their activity. This process called “deconvolution” allows the identification of the most active compounds in a pharmacologically active mixture (Fig. C9). The deconvoluted compounds (concentration of 5 µM per compound) were pre-incubated with the different pseudotypes one hour on ice, and infection assay carried out as described before.

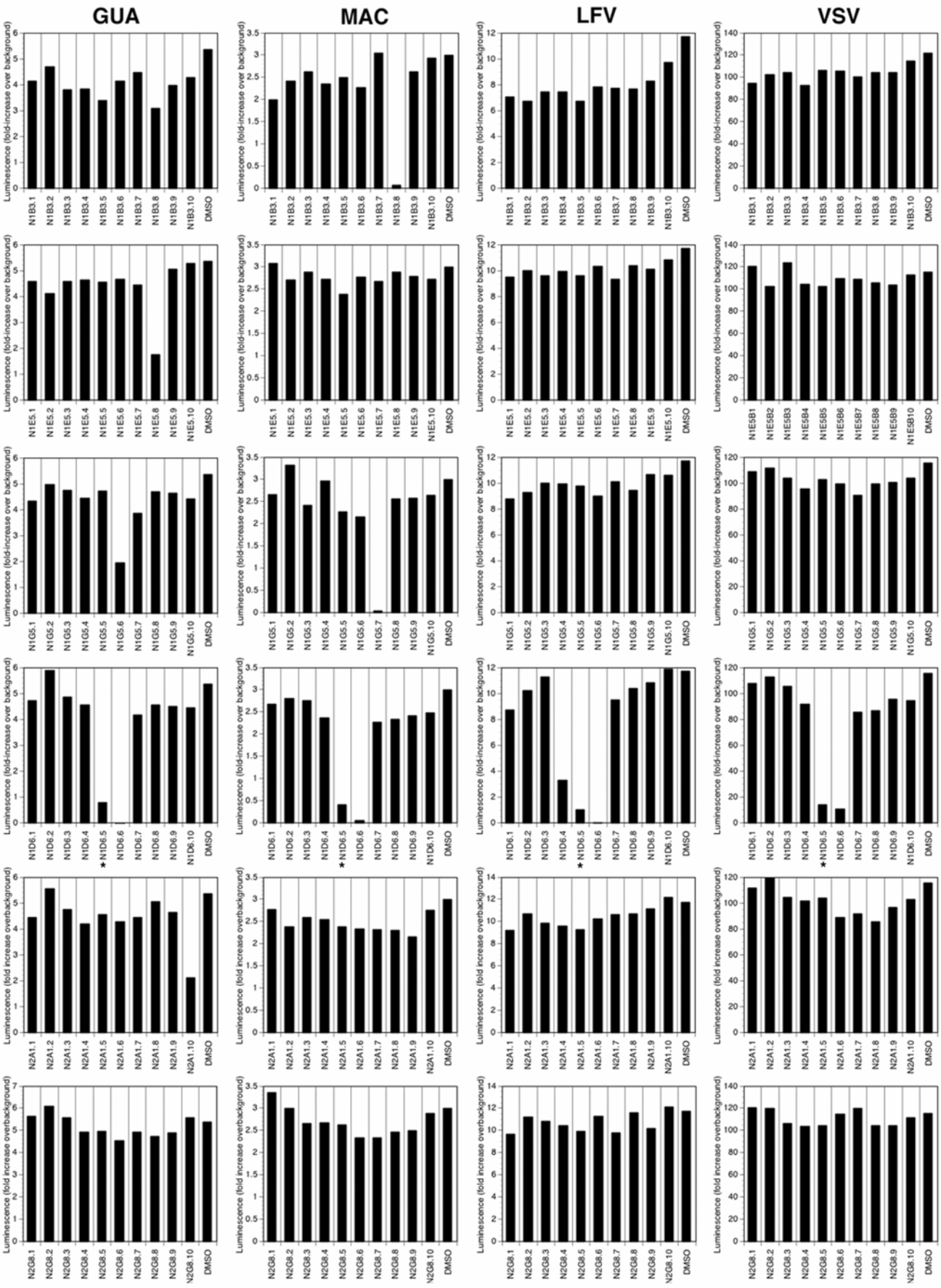
GUA
MAC
LFV
VSV
Luminescence (fold-increase over background)
Luminescence (fold increase overbackground)
N1B3.1 N1B3.2 N1B3.3 N1B3.4 N1B3.5 N1B3.6 N1B3.7 N1B3.8 N1B3.9 N1B3.10 DMSO
N1E5.1 N1E5.2 N1E5.3 N1E5.4 N1E5.5 N1E5.6 N1E5.7 N1E5.8 N1E5.9 N1E5.10 DMSO
N1E5B1 N1E5B2 N1E5B3 N1E5B4 N1E5B5 N1E5B6 N1E5B7 N1E5B8 N1E5B9 N1E5B10 DMSO
N1G5.1 N1G5.2 N1G5.3 N1G5.4 N1G5.5 N1G5.6 N1G5.7 N1G5.8 N1G5.9 N1G5.10 DMSO
N1D6.1 N1D6.2 N1D6.3 N1D6.4 *N1D6.5 N1D6.6 N1D6.7 N1D6.8 N1D6.9 N1D6.10 DMSO
N2A1.1 N2A1.2 N2A1.3 N2A1.4 N2A1.5 N2A1.6 N2A1.7 N2A1.8 N2A1.9 N2A1.10 DMSO
N2G8.1 N2G8.2 N2G8.3 N2G8.4 N2G8.5 N2G8.6 N2G8.7 N2G8.8 N2G8.9 N2G8.10 DMSO

Figure C9: Deconvolution of candidate compound mixtures derived from natural product libraries. Individual compounds (50 µM each) were preincubated with the indicated pseudotypes and then added to HeLa cells. Infection was determined as in Fig. C2. Data shown are single measurements and represent one out of two experiments. The candidate marked with * showed cytotoxicity on the cells.

The results clearly show which compounds are specific and which ones are not. Notably, most compounds showed no activity when deconvoluted. In some mixtures, it was not possible to identify individual compounds responsible for the observed anti-viral activity of the mixture. I other cases, one or two compounds were found to have a significant effect while the rest did not. Notably, in one case, we found that although the mixture showed specificity against arenaviruses but not VSV, two individual compounds seem to have broader activity, also neutralizing VSV.

C.3 Evaluation of polyanionic compounds as anti-arenaviral drugs

Polyanionic compounds have been evaluated as very potent anti-viral drugs against arenaviruses, they were found to be selective inhibitors, whereas the compounds were not inhibitory to cell growth. Dextran sulfate, fucoidan and heparin were active at concentrations of 0.1-2.8 µg/ml and not cytotoxic up to a concentration of 400 µg/ml. At concentrations below the cytotoxicity threshold (inhibition of host cell DNA synthesis), the compounds inhibited the growth of Junin virus in Vero cells, as measured at 24, 48 and 72 hours after infection. The mechanism of action of these compounds was thought to be due to the inhibition of virus binding to the host cells (Andrei and de Clercq, 1990).

C.3.1 Selection of candidate polymers: heparin, HS, DS and fucoidan

Glycosaminoglycans (GAGs) are long unbranched polysaccharides containing a repeating disaccharide unit. GAGs are highly negatively charged molecules, with extended conformation that imparts high viscosity to the solution. GAGs are located primarly on the surface of the cell or in the extracellular matrix. Many viruses utilize GAGs, predominantly heparan sulfate (HS) as receptor or co-receptors, together with other molecules. Heparin and heparan sulfate (HS) are both GAGs of physiological significance. Heparin is a modified (higher sulfated) version of HS and is more potent than HS. Heparin is mainly found as a component of intacellular granules of mast cells lining the arteries of the lungs, liver and skin. HS contains higher acetylated glucosamine than heparin and is mainly localized at basement membranes as components of cell surfaces. If a virus uses HS as a receptor or co-receptor,

their surface glycoproteins (GPs) must contain binding sites for HS. Herpes type 1, herpes type 2, pseudorabies virus, bovine herpesvirus, human cytomegalovirus, respiratory syncytial virus, adeno-associated virus type 2, foot-and -mouth disease virus, vaccina virus, sindbis virus and dengue virus use HS as a receptor.

Some other sulfated polyanionic sugars were also tested for inhibition of virus binding and/or entry. Dextrane sulfate is a long-chain polymer of glucose containing 17-20 % sulfur. It has been used as an anticoagulant and also has been shown to inhibit the binding of HIV-1 to CD4+ T-lymphocytes. Fucoidan is sulfated fucose and has been used as anti-ulcer agent, anticoagulant and antineoplastic agent.

In addition to the anionic polymers already tested, we included a novel type of polymer drug that is currently under development, we will call it polymer X because its chemical composition is still a secret.

C.3.2 Test of heparin, HS, DS, fucoidan and polymer X for anti-viral activity against arenaviruses

The pseudotypes were pre-incubated with increasing concentrations of the polymer-compounds on ice for 45 minutes and then added to monolayers of HeLa cells in 96 well-plates. After one hour incubation at 37°C, 5 % CO_2 the inoculum was removed, cells washed and incubated for 48 h. Infection was quantified using the Steady-Glo® high sensitivity luciferase reporter gene assay (Promega) in a Berthold® 96 well-plate luminometer. As shown in Fig. C10, polymer X showed a powerful inhibition of infection by all pseudotypes of LCMV(ARM), LCMV(Cl-13) and, LFV. The polyanionic sugars dextrane sulfate and fucoidan showed some anti-viral activity. The soluble GAGs HS and heparin showed no blocking of pseudotype-infection. This is inconsistent with previous findings (Andrei and de Clercq, 1990) that claim that sulfated polysaccharides are potent and selective inhibitors of enveloped DNA and RNA viruses, were the mechanism of action of is due to the inhibition of virus binding to the host cell.

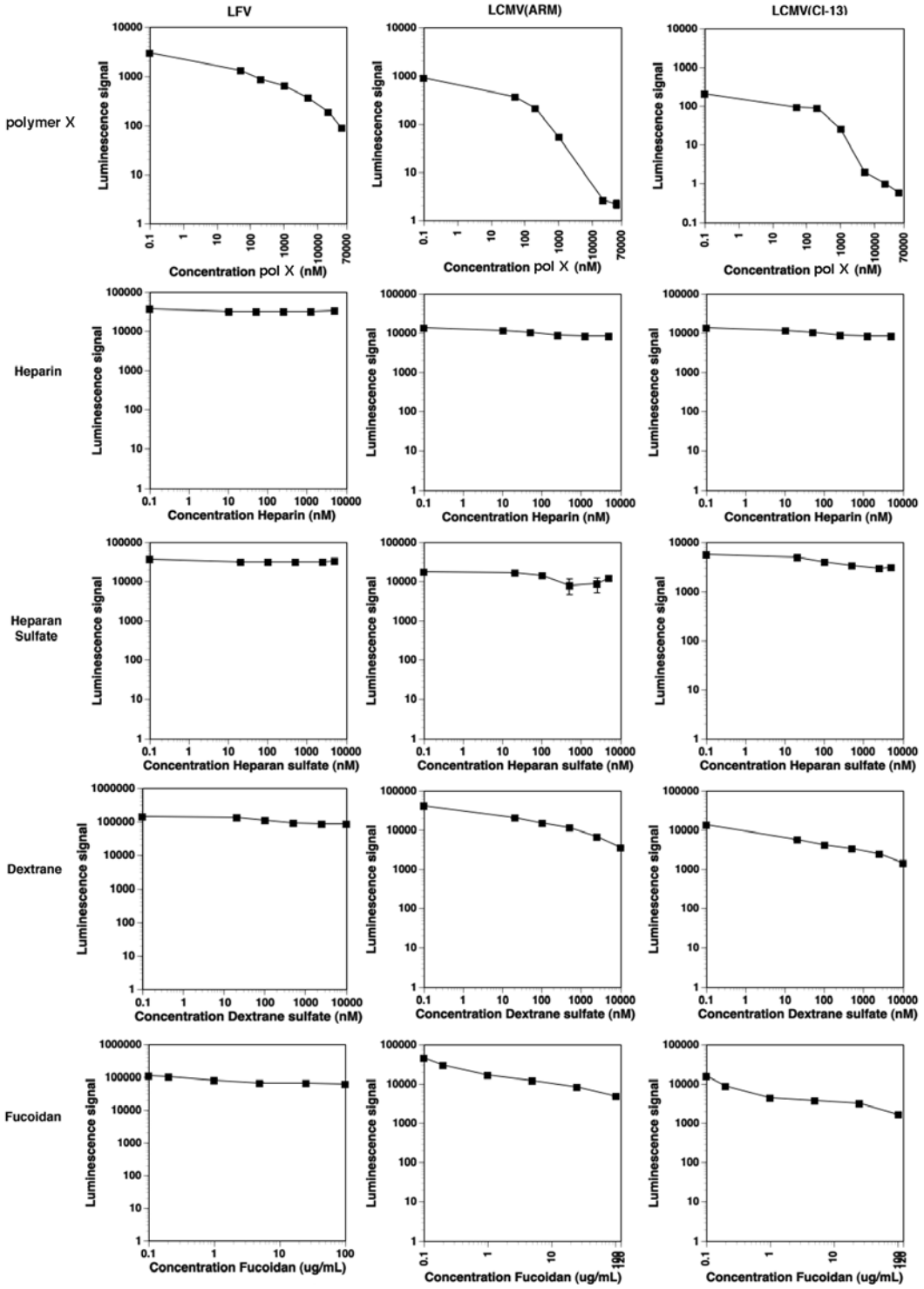

Figure C10A: Blocking of infection of cells with retroviral pseudotypes of LCMV and LFV with polyanionic substances. Pseudotypes (MOI = 1) were pre-incubated with the indicated concentrations of polymer X, heparin, heparan sulfate, dextrane sulfate, and fucoidan for one hour on ice. Pseudotypes were then added to monolayers of Vero cells in 96 well-plates. After 48 h, infection was quantified using the Steady-Glo® luciferase reporter gene assay as in Fig. C2. Luminescence is expressed as fold-increase over uninfected controls (n = 3 $\pm$ SD). Note that the y-axis is a log scale.

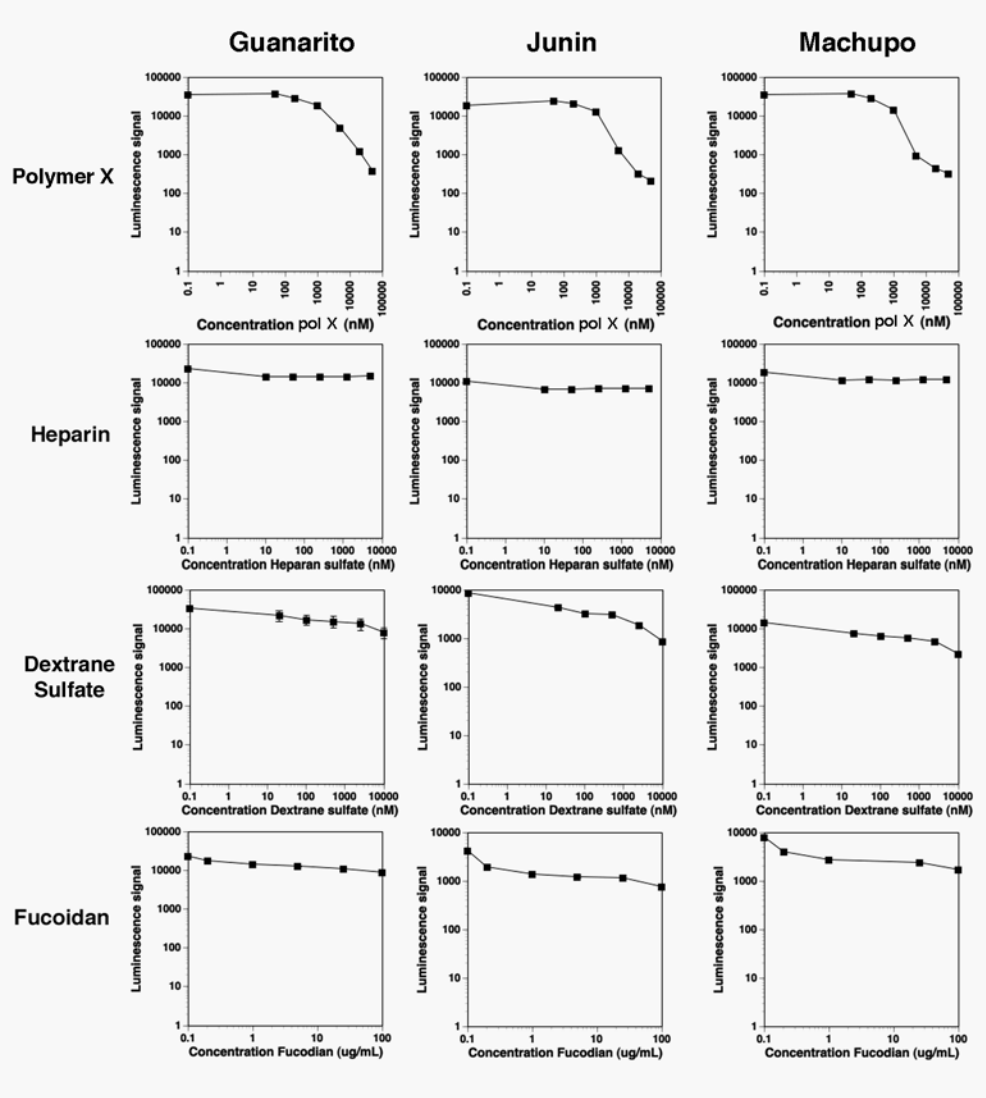

Figure C10B: Blocking of infection of cells with retroviral pseudotypes of Guanarito, Machupo, and Junin with polyanionic substances. Pseudotypes (MOI = 1) were pre-incubated with the indicated concentrations of polymer X, dextran sulfate, heparin, and fucoidan for one hour on ice. Pseudotypes were then added to monolayers of Vero cells in 96 well-plates. After 48 h, infection was quantified as in Fig. C10A.

C.3.3 What is the role of HS and glycosaminoglycans in arenavirus infection?

To address the role of heparan sulfate in the infection of cells with arenaviruses, we made use of the mutant CHO cell line pgsA-745 that is deficient in UDP-D-xylose: serine-1, 3-D xylosyltransferase and lacks all glycosaminoglycans (Esko et al., 1985). PsgA-754 and wild-type CHOK1 cells were infected with pseudotypes of LCMV (ARM53b, cl-13), LFV, Guanarito, Junin, and Machupo, as well as VSV-pseudotypes that do not depend on glycosaminoglycans for infection. As shown in Fig. C11, pgsA-745 that lack all glycosaminoglycans are more efficiently infected than normal CHOK1 cells,

indicating that glycosaminoglycans slightly inhibit infection. In contrast, absence of glycosaminoglycans resulted in a mild reduction in infection with LCMV pseudotypes, while the South American HF virus pseudotypes infected both cell types, pgsA-745 and CHOK1 with similar efficiency. Together, these data indicate only minor differences in infection of cells by arenaviruses in presence or absence of glycosaminoglycans, excluding an essential role as a receptor or co-receptor.

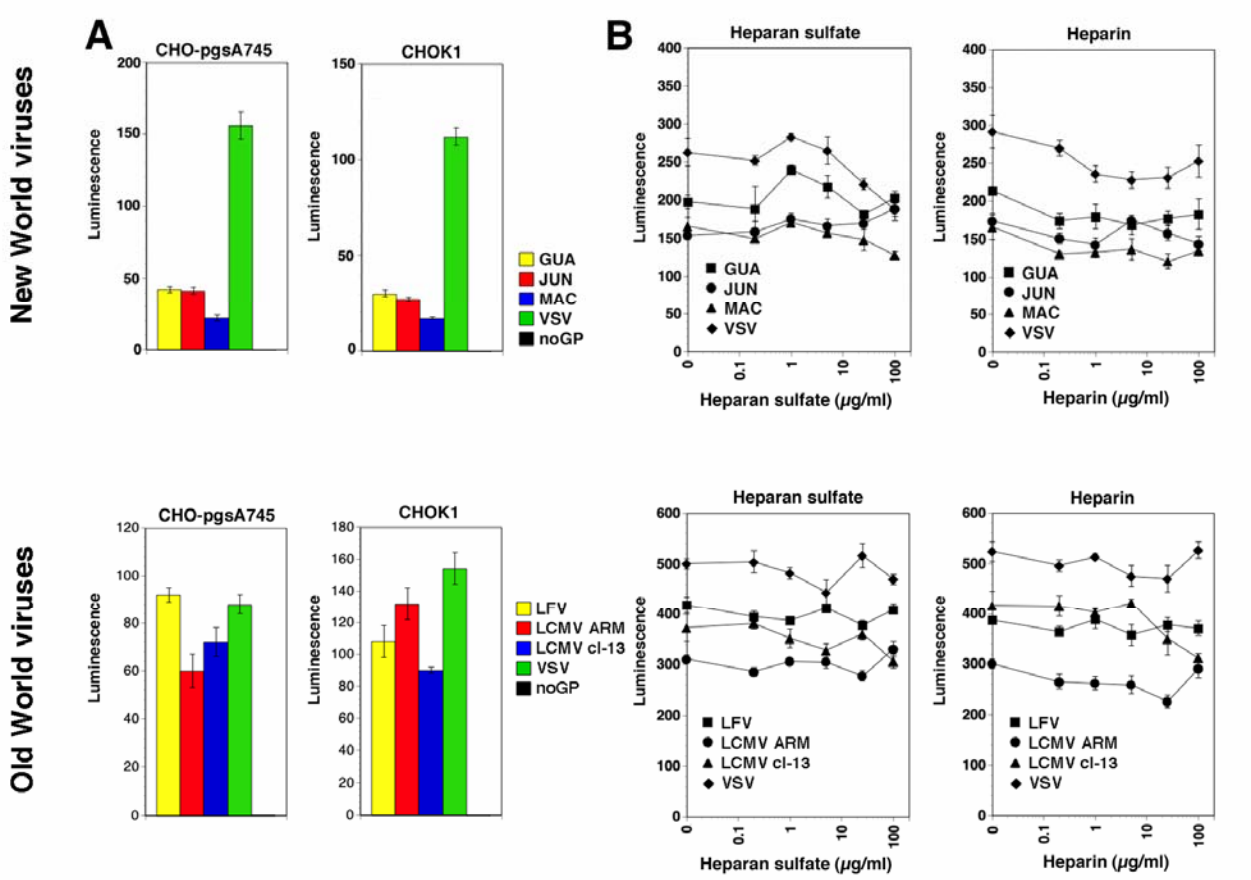

Figure C11: Infection of arenaviruses is not dependent on glycosaminoglycans. (A) Infection of glycosaminoglycan-deficient and wild type CHO cells with pseudotypes: Glycosaminoglycan-deficient CHO mutant psgA-745 cells and CHOK1 cells were infected with the indicated pseudotypes, at an MOI of 1. Infection was quantified after 48 hours by luciferase assay (n = 3; ± SD). (B) Blocking of pseudotype infection with heparan sulfate and heparin: Pseudotypes were pre-incubated with the indicated concentrations of heparan sulfate and heparin for one hour on ice and then added to monolayers of Vero cells (MOI of 1). Infection was quantified after 48 hours by luciferase assay as in A (n = 3; ± SD).

C.3.4 Evaluation of polymer X as a drug against LCMV

The prototypic arenavirus lymphocytic choriomeningitis virus (LCMV) is a powerful experimental model that provided novel concepts in immunology and virology that have been extended to other viruses, bacteria, and parasites. Investigations using the LCMV model have also uncovered the ability of non-cytolytic persistent viruses to avoid elimination by the host immune responses, and to induce disease by interfering with specialized functions of infected cells, revealing a new way by which viruses do harm in the absence of the classic hallmarks of cytolysis and inflammation. Moreover, there is increasing evidence that LCMV might be a neglected human pathogen with significant clinical implication. These findings together make LCMV persistent infection of the mouse a very attractive model system to develop and evaluate therapies to treat acute and chronic viral infections and associated disorders. Since no firm data on anti-viral effects of polymer X on LCMV are available at present, we evaluated the anti-viral potential of polymer X on live LCMV virus. First, we studied the ability of polymer X to block infection of VeroE6 cells with the prototypic LCMV isolate LCMV ARM53b. For studying the anti-viral activity of Polymer X against LCMV in a quantiative manner, we used FACS to determine viral infection. The virus was pre-incubated with increasing concentrations of the polymer-compounds on ice for 45 minutes and then added to monolayers of Vero cells in 6 well-plates. After one hour incubation at 37°C, 5 % CO_2 the inoculum was removed, cells washed twice, complete medium were added and plates were incubated for 20 h. After incubation, the cells were washed twice, detached from the plate by trypsin treatment, and re-suspended in 1% FBS/PBS. Cells were washed and fixated in 4% (w/v) PFA/PBS for 10 min at room temperature. Cells were washed twice, re-suspended with primary antibody and incubated for 45 min on ice in the dark. After two additional wash steps, cells were re-suspended with secondary antibody and incubated for 45 min in the dark. The cell-suspention was washed and re-suspended in PBS and kept in the dark before measured on the flow cytometer.

In several independent experiments we observed a significant reduction of LCMV infection with an IC50 of 26 ($\pm$ 7) nM (Fig. C12A). A similar reduction of infection was observed with the prototypic immunosuppressive LCMV isolate LCMV cl-13. In a control experiment, the activity of polymer X against vesicular stomatitis virus (VSV) was tested using a recombinant VSV expressing a green fluorescent protein marker (VSV-GFP). VSV-GFP was pre-incubated with polymer X and subsequently added to VeroE6 monolayers. Infection was quantified by detection of GFP in FACS. In contrast to the marked reduction of LCMV infection after pre-incubation with polymer X, no significant decrease in VSV-GFP infection was observed (Fig. C12B). Cell viability was verified by

staining with 7-amino-actinomycin (7-AAD) and no significant cytotoxicity was observed upon exposure of cells to up to 20 µM polymer X. Together, these data demonstrate a potent and significant anti-viral activity of polymer X against LCMV but not VSV-GFP.

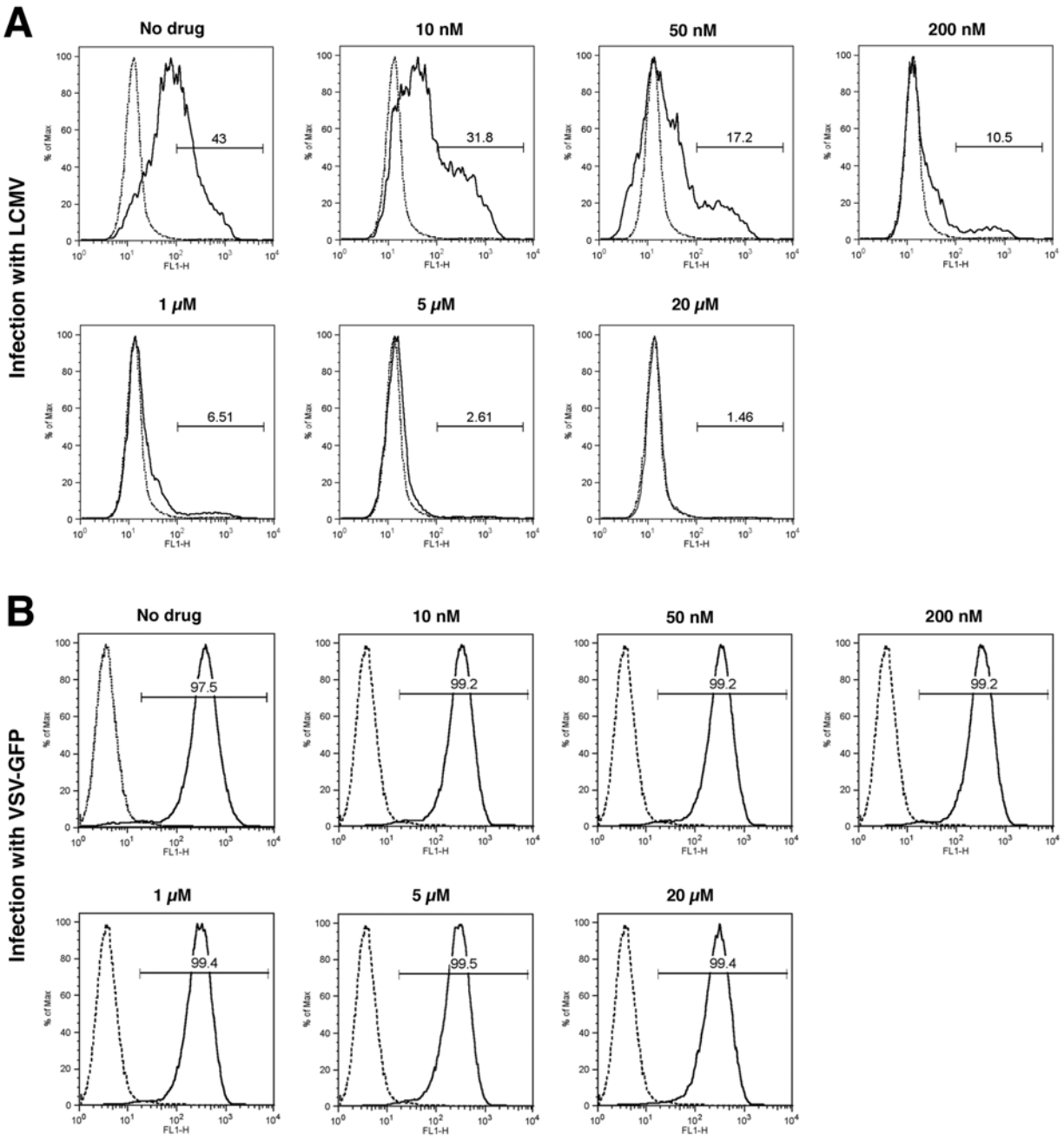

Figure C12: Blocking of virus infection with polyer X. Blocking of infection with LCMV ARM53b (A) and VSV-GFP (B): Virus was pre-incubated with the indicated concentrations of polymer X for one hour on ice and then added to monolayers of VeroE6 cells (MOI = 3). After 24 hours, cells were briefly trypsinized, detached and single cell suspensions prepared. LCMV infection was detected by intracellular staining for LCMVNP. Cells were fixed, permeabilized, and stained with mAb 113 anti-LCMVNP and a FITC-labeled secondary antibody. Infection with VSV-GFP was detected by direct fluorescence excitation of GFP. For flow cytometry, a

FACSCalibur® flow cytometer was used. In histograms, the y-axis represents cell numbers and the x-axis fluorescence intensity (FL1-H for FITC/GFP). The dotted line represents uninfected controls and the solid line infected samples. Percentages of NP or GFP positive cells are given. One representative experiment out of three is shown.

To determine the step in LCMV infection blocked by polymer X, the drug was added at different time points relative to infection. LCMV ARM53b was used at MOI of 0.01, 0.1, and 1. Polymer X (2 µM) was added exactly at the time of infection, 6 hours post infection, or 24 hours post infection. Infection levels were assessed after a total infection period of 48 hours by determination of LCMVNP expression using immunofluorescence staining with mAb 113 anti-LCMVNP (Fig. C13A) or FACS (Fig. C13B). Vero cells were cultivated in 8 well LabTek chamber slides and infected with LCMV(ARM53b) at MOI of 0.1. For one sample, LCMV were pre-incubated with the polymer X one hour on ice before added to the cells. For the other samples, polymer X was added at different timepoints after infection. After a total infection time of 48 hours, the medium/virus/polymer X solution removed and cells fixed at 37°C for 15 min in the dark. Cells were washed twice and incubated with PBS/1%(v/v) FCS for 15min at room temperature. The cells were so incubated 15 min with PBS/1%(v/v) FCS/0.1% (w/v) saponin. Primary antibody mAb 113 anti-LCMVNP was added and cells incubated for 1 hour at room temperature in the dark. Cells were washed twice and incubated with a FITC-conjugated secondary antibody for 45 min at room temperature in the dark. The cells were so washed three times before 1 drop of Vectashield were added to each fixing square and the cells covered with a coverglass. Images were acquired using a fluorescence microscope.

As shown in Fig. C13, the data strongly suggest that the polymer drug blocks an early step in viral infection. When added at the time of infection, polymer X efficiently blocked infection of LCMV at all MOI tested, indicating fast inhibition of an early step in infection. Addition of polymer X after 6 or 24 hours resulted in marked decrease in the number of infected cells, indicating efficient blocking of cell-to-cell spread. However, the similar levels of LCMVNP expression (mean immunofluorescence intensities) in infected cells in presence and absence of polymer X indicate that the drug has little effect on LCMVNP expression once infection has occurred (Fig. C13B).

Together, the data show that polymer X has a specific anti-viral activity against prototypic isolates of LCMV. The drug efficiently blocks initial steps of virus infection and prevent cell-to-cell spreading of virus. That means that polymer X is active in an early step of the virus lifecycle. The efficient neutralization of the retroviral pseudotypes of LCMV indicates further that the viral GP is the main drug target.

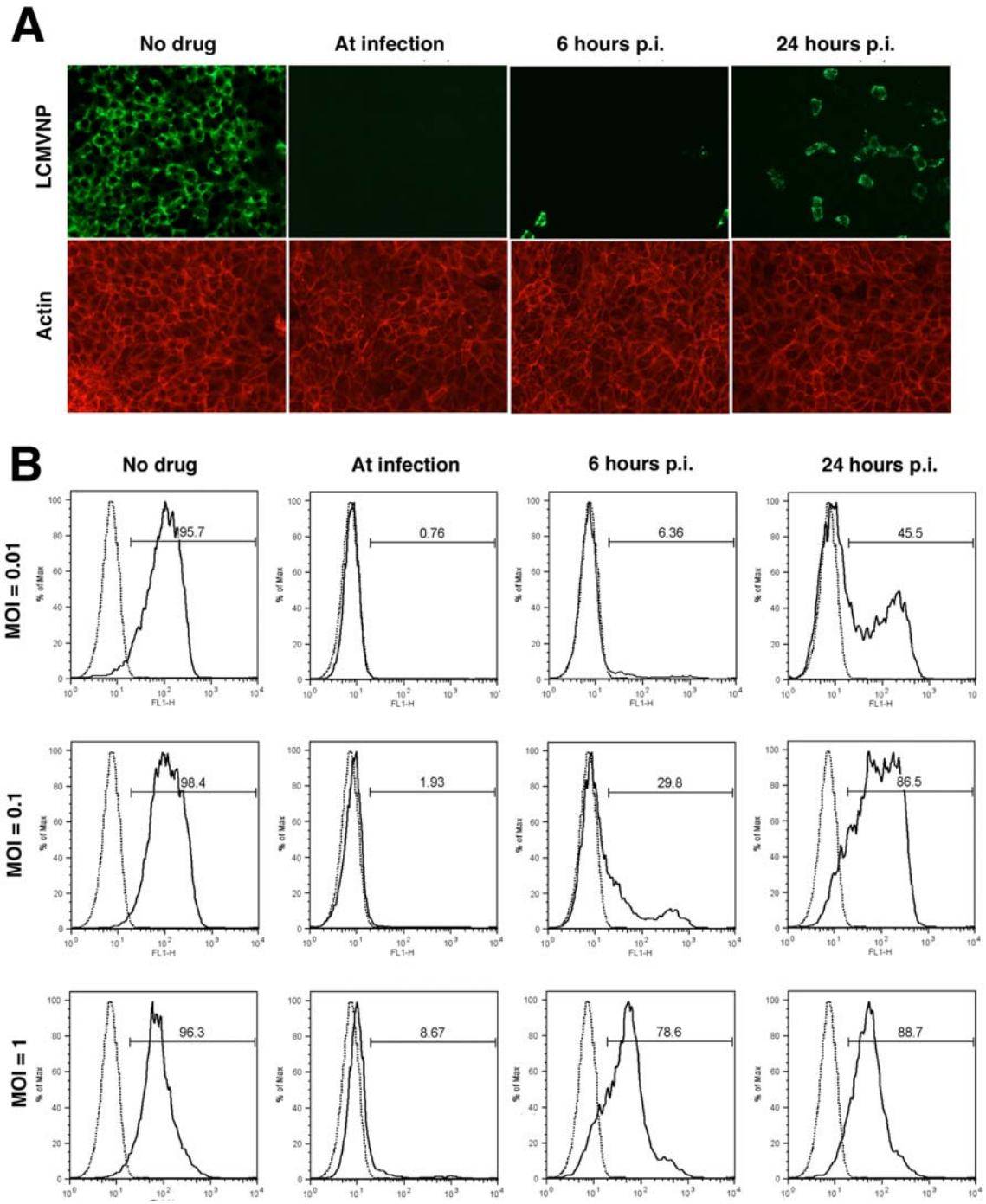

Figure C13: Polymer X blocks infection and cell-to-cell spread of LCMV: (A) Detection of virus infection and propagation by immunofluorescence. Monolayers of VeroE6 cells were infected with LCMV ARM53b at MOI of 0.1 and polymer X added at the indicated time points. After 48 hours, cells were fixed, permeabilized and stained with mAb 113 anti-LCMVNP using a FITC-conjugated secondary antibody and phalloidin-rhodamine for counterstaining of F-actin filaments. (B) Monolayers of VeroE6 cells were infected with LCMV ARM53b at MOI of 0.01, 0.1, and 1 and polymer X added at the indicated time points. After 48 hours, infection

was determined by FACS staining for LCMVNP as in Fig. C12. The dotted line represents uninfected controls and the solid line infected samples. Percentages of NP positive cells are given.

C.4 Production of recombinant Lassa fever virus glycoprotein

The glycoprotein (GP) of Lassa fever virus (LFV) serves as virus attachment protein to its receptor on host cells. The GP precursor (GP-C) is post-translationally cleaved into GP1, which is implicated in receptor binding and GP2 that is structurally similar to the fusion active membrane proximal portions of the GPs of other enveloped viruses. In my studies I have identified a number of small molecule compounds and polymer drugs that block infection of human cells by retroviral pseudotypes containing the GP of LFV (LFVGP). Among the candidate drugs, compounds and substances that target the viral GP are of greatest interest since they are able to target the free virus and will most likely not interfere with crucial cell function, which will minimize their potential for adverse side effects.

As pointed out in the introduction (section A.1.3), the viral GP at the surface of free LFV virions is in its metastable pre-fusion state. These pre-fusion state GP trimers are the functional units of target cell recognition, entry and fusion in arenaviruses. The production of a stable, trimeric form of LFVGP in its pre-fusion state is therefore of paramount importance regarding the identification of anti-viral drugs targeting the GP.

To prevent the transition from the (metastable) pre-fusion into the energetically more stable post-fusion complex, the GP2 subunits containing the fusion machinery have to be locked into a stable trimer that does not allow significant conformational changes. Recent attempts to express recombinant influenza hemagglutinin (HA) derived from the influenza strain of the 1918 pandemic faced the problem that the HA did not form trimers (Stevens et al., 2004). Among the potential trimerization domains tested by Stevens and colleagues, the recently identified foldon domain derived from bacteriophage T4 fibritin (Tao et al., 1997) turned out to be very efficient in the induction of trimer formation. The foldon domain (Fig. C14), spans the C-terminal residues G457 to L483 of the bacteriophage T4 fibritin and forms a trimeric complex of very high stability. Based on its properties as a powerful driving force for trimerization, we will construct fusion proteins of the ectrodomain of LFVGP and the foldon-domain (Fig. C15). These LFVGP-foldon constructs will be tested for trimer formation and receptor binding. Trimers formed by the LFVGP-foldon fusion proteins may be powerful tools for drug screening and for the investigation of the virus-cell interaction.

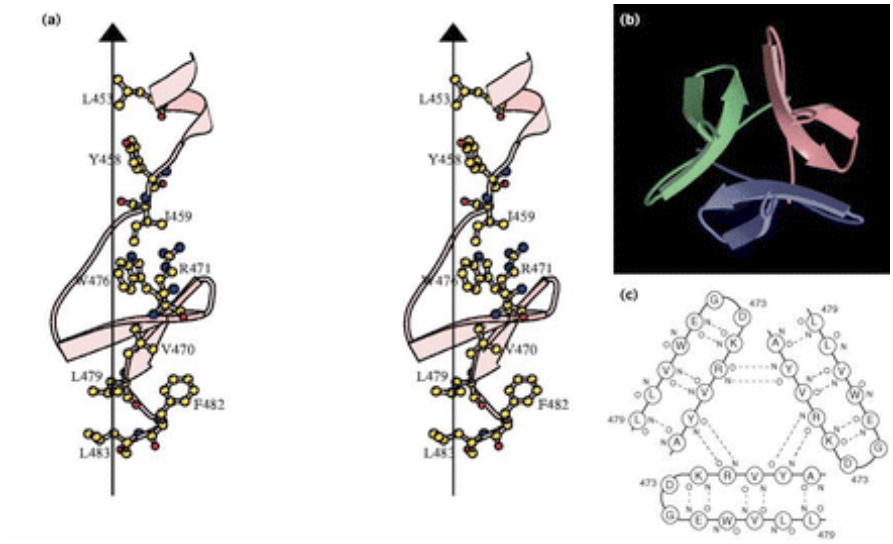

Figure C14: Structure of the foldon domain of bacteriophage T4 fibritin (from Tao et al., 1997). (a) Stereo diagram of the C-terminal foldon domain of a fibritin E subunit. The sidechains shown are those located in the hydrophobic interior formed at the interface between the subunits. (b) Ribbon diagram of the foldon domain looking along the trimer axis. (c) Hydrogen of the main chain formed within the foldon domain.

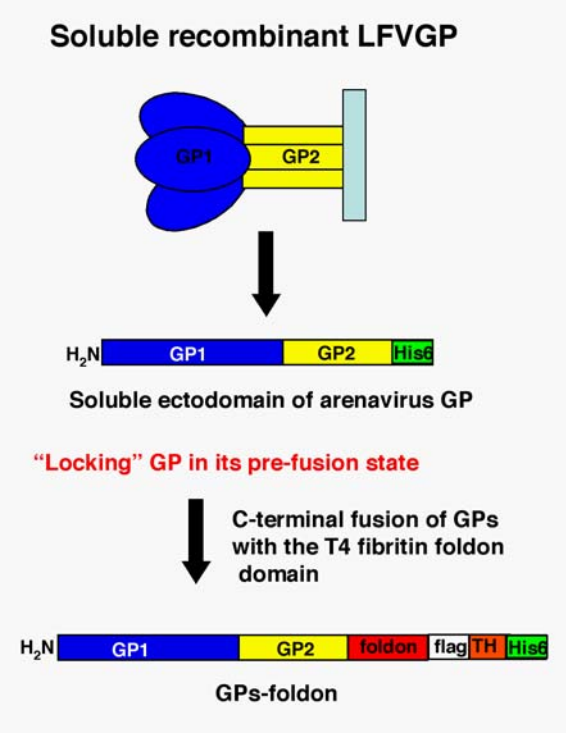

Figure C15: Design of a soluble trimeric form of LFVGP locked in its pre-fusion state. For details see text.

C.4.1 Design of the LFVGP-foldon fusion protein

Layout of the foldon cassette:

The T4 fibritin foldon domain **(**GYIPEAPRDGQAYVRKDGEWVLLSTFL)
corresponds to residues G457 to L483 of the bacteriophage T4 fibritin (Tao et al., 1997).
The fusion of the hole sequence of LFVGP from M(1) to LGLV(431) derived from LFV Josiah with the foldon domain will include a GGGS linker between the LFVGP and the foldon domain. In addition, we will add a flag-tag (DYKDDDDK), a throbin cleavage site (LVPR/GS), and a six His tag that allows purification of the soluble recombinant protein by Ni-NTA affinity chromatography.

Design of the LFVGP-foldon fusion protein:

M(1)…LGLV(431)**GGGSGYIPEAPRDGQAYVRKDGEWVLLSTFLDYKDDDDK LVPR/GS HHHHHH**Stop

L G L V(431)
5’- TTG GGT CTA GTT GGC GGT GGA TCC GGC TAC ATC CCC GAG GCC CCG CGC GAC

GGC CAG GCC TAC GTG CGC AAG GAC GGC GAG TGG GTG CTG CTG TCC ACC TTC CTG

GAC TAC AAA GAC GAT GAC GAT AAA CTT GTC CCA CGT GGT TCT CAT CAC CAT CAC

CAT CAC TAG

Flag: 5’- GAC TAC AAA GAC GAT GAC GAT AAA-3’
D Y K D D D D K

Thrombin cleavage side: CTT GTC CCA CGT GGT TCT
L V P R / G S

A cDNA sequence containing the foldon cassette, followed by the flag-tag, thrombin cleavage site, and His-tag has previously been made, subcloned into a basis vector (pBluescript), and verified by double strand DNA sequencing. For the construction of the fusion proteins with LFVGP, the corresponding sequence of LFVGP M(1)… LGLV(431) derived from LFV Josiah were amplified by PCR using the cDNA of full-length LFVGP as a template as described in M&M.
The fragment from LFVGP pcAGGs cDNA was amplified in PCR with the forward primer at position 1130 and the backward primer LFEb that makes a blunt, phosphorylated 5’ end at position 1348. This fragment contains a restriction-site, KpnI, at position 1164 that was used for cutting the fragment. The

Fol 1 pBS was cut with enzymes at restriction-sites for NaeI and XhoI. The pBS was cut open with restrictionenzyme KpnI and XhoI. The PCR fragment and the Fol 1 pBS were ligated into the open pBS, that gives LFVGP-Fol 1 pBS. Then the missing fragment from LFVGP pcAGGs was cut out with enzymes NotI and KpnI (position 1164). The assembled fragment from LFVGP-Fol 1 pBS was cut out with enzymes KpnI (position 1164) and ApaI (position 1209). These two fragments were ligated into pcDNA3.1 (pino) that is cut opend with enzyme NotI and ApaI. This made LFVGP-Fol 1 pcDNA (pino) that were expressed in HEK293 cells.

C.4.2 Expression and characterization of the GP1-foldon fusion proteins

The LFVGP-foldon fusion proteins were expressed in HEK293 cells under serum-free conditions and the LFVGP-foldon fusion proteins isolated by pull-down from conditioned cell culture supernatant by NiNTA affinity chromatography. Protein solution was centrifuged and the supernatant removed. Pellet were eluted in 1 x SDS-PAGE loading reducing buffer and separated on a 12% Tri-Gly gel. Expression was tested by Western-blot analysis (Fig. C16). From the blot we can see that the LFGP-foldon fusion is efficiently secreted and that the major part of our construct gets cleaved

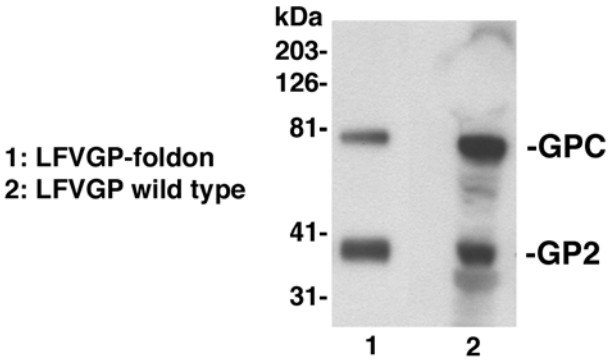

Figure C16: Detection of recombinant LFVGP in Western-blot. LFVGP-foldon isolated from cell culture supernatants by NiNTA pull-down (1) and total cell lysate of HEK293T cells transfected with the full-length wild-type LFVGP containing a C-terminal flag-tag (2) were separated by SDS-PAGE, blotted to nitrocellulose and probed with mAb M2 to flag tag. Molecular masses (kDa) and the positions of the GP precursor (GPC and mature GP2 are indicated).

D. DISCUSSION

The goal with this study is to find a drug that can fight Lassa fever that affects an estimated 180 million individuals in Western Africa. There is neither an efficient cure nor an efficacious vaccine for LFV. With over 200 000 infections per year and several thousand deaths, LFV represents a severe threat for human health and a major humanitarian problem. Ribavirin is so far the only drug for patients infected with LFV. Ribavirin does not neutralize free virus, but works in the late steps of the virus lifecycle. The goal is to develop drugs that can act as "gatekeepers", i.e. drugs that are able to block the early step of the virus lifecycle, the virus-receptor binding and/or entry. To block the virus binding and/or entry and then cell to cell spread, will give the infected patients immune system a better chance in generating an efficient anti-viral immune response.

We tested small molecule derived from combinatorial chemical libraries to see if there was any blocking of the virus-receptor interaction. We also tested sulfated polysaccharides like dextrane sulfate, fucoidan, heparan sulfate and heparin that have showed anti-viral activity in studies already done. In addition, we tested polymer X that is an anionic polymer that has showed activity against HIV.

D.1 Discovery of novel small molecule inhibitors of Lassa fever virus (LFV) infection from combinatorial chemical libraries and natural product libraries

To identify potential anti-viral compounds, small molecule inhibitors of protein interaction derived from combinatorial chemical libraries were screened. This strategy has been used to identify several physiologically active inhibitors of novel biochemically not yet characterized interactions. The compounds/compounds mixtures were screened for their ability to block retroviral pseudotype infection in target cells. Retroviral pseudotypes contain LFVGP in their envelope and a luciferace reporter gene in their genome. The luciferase reporter gene makes the pseudotypeinfection easy to detect. Since live LFV is a BSL4 pathogen, studies with virus are restricted to BSL4 laboratories. The pseudotypes allow us to do our work under BSL2 conditions.

Many studies on receptor-ligand binding indicate that in most cases, small clusters of amino acid recidues mediate the energetically most important interaction. Using Dr. Boger's libraries, erythropoieitin (EPO) mimetics (promote EPO receptor dimerization) (Goldberg et al., 2002), inhibitors of Myc/Max dimerization (Berg et al., 2002), inhibitors of androgen receptor binding to DNA (Boger et al., 2000a), inhibitors of LEF-1/β-catenin dimerization (Boger et al., 2000b), and

inhibitors of the binding of the protease MMP-2 to αvß3 integrin have been discovered (Silletti et al., 2001; Boger et al., 2001). That makes us think that small molecules, interacting with the reseptor-binding site for the virus, can block virus infection in cells.

We screened combinatorial libraries of small molecules that consist of imidodiacetic acid-based, pyrrolidine-based peptidomimetic but also heterolytic compounds. The compounds were not attached to solid supports making them immediately available for binding or functional assays. I tested 13 complete libraries. Each library had 80 wells that contained single compounds or mixtures of 4-10 compounds (that made the screening more effective). Totally this gave a number > 10,000 compounds. Duplicate screening resulted in the identification of circa 4-5% compounds/mixtures that showed reproducible reduced LFV-PS infection by > 50%. To test our candidate for specificity, we screened the compounds for inhibition of VSV-pseudotype infection. VSVGP is structurally unrelated to LFVGP and binds to a different receptor. If the compounds are specific, they should not affect the infection of target cells by VSV-pseudotype. My screening together with previous screenings done in the lab of another 31 libraries, corresponding to > 50, 000 compounds yielded 157 (4.5%) compounds/mixtures that showed reproducible reduced LFV-PS infection. Determination of target specificity revealed that 32 single compounds and 53 compound mixtures out of a total of 125 specifically reduced infection in LFV-PS but not VSV-PS. We found difference in specificity between different libraries; some were very consistent, with most candidate compounds being specific for inhibition of LFV-GP infection, when other compounds showed effect both against LFV-PS and VSV-GP.

A selection of 17 individual compounds was then further characterized. The selection was made based on the extent of inhibition, degree of specificity, and also chemical considerations, raised by our chemist collaborators. The selected compounds were tested for inhibition of pseudotype infection in different cell-lines (He-La, A549 and VeroE6) that showed that the candidate compounds had consistent blocking of LFV-PS infection in all cell lines and were independent of the specific target. The determination of the dose-response of the individual compounds was also tested on the different cell lines. We found 8 candidate compounds to be particularly good selective inhibitors against LFV-PS. Many of these candidate compounds were also active against the human pathogenic South American HF viruses Guanarito, Junin and Machupo. The responses to the compounds in the different NWAV-PS were very similar as in LFV-PS. Structurally our candidate compounds were hydrophobic molecules with a high degree of aromaticity. Comparison of the most active structures revealed that an overall tripartite symmetry was a common structural theme. Examples are shown in Fig. D1.

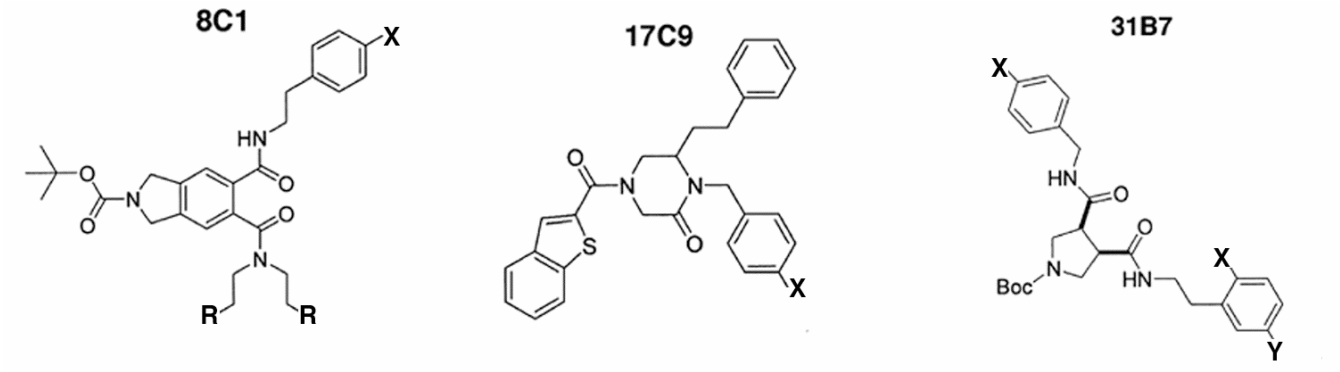

Figure D1: Representative examples of candidate compounds

Our candidate drugs may block one or several of the following steps: virus-receptor binding, binding of the viral GP to a yet unknown co-receptor, virus internalization by vesicles, and pH-dependent fusion of the late endosome membrane with the viral membrane that releases the virus into the cytoplasm (Fig. D2). More or less modified, these compounds are small and an oral administration of the drug is therefore possible (as far as the stability and absorption in the GI-tract not cause any problems). An oral route of administration would be of importance for better viability for the patients when this does not need any specialized assistance.

D.2 Evaluation of polyanionic compounds as anti-arenaviral drugs

We tested polyanionic compounds as anti-arenaviral drugs because they have from earlier studies showed antiviral activity against arenaviruses. They were found to be selective inhibitors, whereas the compounds were not inhibitory to cell growth. In Graciela Andrei and Erik De Clercqs paper (1990) it is claimed that the polyanionic compounds worked at the early step of infection of arenaviruses, by inhibiting reseptor binding and/or fusion and entry. In general poly-anionic compounds have showed activity against HIV-1 and other viruses. The anti-HIV-1 activity decreased with increasing the hydrophobicity (Clercq et al, 1995). In our study, we tested heparin, heparin sulfate (HS), dextrane sulfate and fucoidan. We selected those because many viruses utilize glycosaminoglycans, predominantly HS as receptor or co-receptors, together with other molecules. If a virus use HS as a receptor or co-receptor, their surface glycoproteins must contain binding sites for HS. By adding the glycosaminoglycans to the viruses, the binding site for glycosaminoglycans will be occupied and the virus will not be able to infect the target cell. Our results showed that dextrane sulfate and fucoidan had some anti-viral activity. Heparin and HS did not show any significant anti-viral activity. The

results did not come out as we expected them from the study already done (Andrei and de Clercq, 1990). The glycosaminoglycans did not show any significant anti-viral activity on the receptor binding/fusion/entry stage. The glycosaminoglycans must therefore have a different mechanism of action than Clercq et al claimed. The glycosaminoglycans antiviral-effect may have a mechanism related to a later step in the virus life-cycle than the steps we tested (virus receptor binding/fusion/entry).

We can suggest a general model for the (normally weak) anti-viral effect of polyanionic compounds and the increased infection often seen after application of polycations. Increased infection after application of polycations is caused of the decrease in repulsions between the viruses. Polycations are of the opposite charge than the surface of the virus particles that are negatively charged (because of the sugars attached). Polycations will therefore lower the surface energy of the viruses so they can easier go together in clusters. A cluster of viruses will then be able to enter the cell through one receptor instead of one single virus. We will then see an increased infection.

If we look at the different activities of heparin/HS vs. dextrane sulfate/fucoidan, and polymer X, it seems like it is not only the charge of the polymer that is responsible for anti-viral activity. The hydrophobicity of the compound is also of importance. The more hydrophobic the compound is, the better anti-viral activity. Polymer X shows broad anti-viral activity. It shows nearly full inhibition of infection of all the pseudotypes. Polymer X has the same charge as the polyanionic compounds, but is more hydrophobic. Tested on real virus (LCMV and VSV), the polymer X shows specificity. Polymer X acts at the step of infection. If it blocks the receptor-binding, fusion and/or entry is still unknown. Since the polymer X act at the step of infection, it can inhibit cell-to-cell spread. The polymer X should for that reason be used early in a virus-infection. Anyway, if cells are protected against re-infection in an infected individual, the immune system will have a greater opportunity to clear the infection. Polymer X is a huge molecule; it is too big to be absorbed in the GI-tract and must be administrated i.v. When the mechanism of action is understood for polymer X and the small molecule drugs, it is a possibility to combine the two drugs to make a better anti-viral effect.

D.3 Production of recombinant Lassa fever virus glycoprotein

Among the candidate drugs, the compounds and substrates that target the viral GP are of greatest interest since they are able to target the free virus and will most likely not interfere with crucial cell function, which will minimize their potential for adverse side effects. The viral GP at the surface of free LFV virions is in its metastable pre-fusion state. These pre-fusion state GP trimers are the

functional units of target cell recognition, entry and fusion in arenaviruses. The production of a stable, trimeric form of LFVGP in its prefusion state is therefore important regarding the identification of anti-viral drugs targeting the GP. It is critical to have the GP in its prefusion state, because this is the state where the drugs have to be active (before infection of the cell). The conformation of the GP is changed in its post-fusion state and the drugs may not recognize this conformation and the study will then not be correct. To prevent the transition from the (metastable) pre-fusion into the energetically more stable post-fusion complex, the GP2 subunits contain the fusion machinery have to be locked into a stable trimer that does not allow significant conformational changes. These LFVGP-foldon constructs will be tested for trimer formation and receptor-binding. Someone else has already done a similar approach (Eschli et al., 2006) and that worked out well. Our approach worked out as expected. We got expression of LFVGP-foldon and 80% of the constructs were cleaved. Preventing this cleavage by mutate its cleavage site, can possible give the construct more stability.

D.4 Outlook

My work at Scripps resulted in the identification and first characterization of a number of candidate small molecules that show specific anti-viral activity against pseudotypes of LFV and other human pathogenic arenaviruses and in the demonstration of a potent anti-viral effect of a novel polymer drug against LCMV. My results provide the basis for the following studies that will further characterize and optimize our drug candidates.

D.4.1 Further validation of candidate compounds

Determination of the drug target: To avoid undesired side effects of our small molecule drugs, we will select for compounds that efficiently block LFV infection by specifically targeting the viral GP but not cellular receptor molecules. For this purpose, the recombinant soluble form of LFVGP, which I made (Results, section C4) will be used.

Validation of activity of candidate drugs against live LFV: Activity of candidate drugs against live LFV will be tested in a BSL4 facility by Dr. Christina Spiropoulou from the Special Pathogens Branch of the Centers for Disease Control and Prevention (CDC) in Atlanta, GA. The Special Pathogens Branch has also a well-established small animal model (guinea pig) for LFV infection for later *in vivo* testing of candidate compounds.

D.4.2 Determination of the mechanism of action of candidate drugs

Once we have identified specific compounds that block infection of human target cells with LFV in an efficient manner, we will start to investigate the exact mechanism of their activity. The drugs could target the following steps or combinations thereof: 1) the binding site(s) on LFVGP for its cellular receptor(s), 2) the pre-fusion conformation of LFVGP as present at the virion surface, and 3) A conformational intermediate of LFVGP occurring during pH-dependent membrane fusion (Fig. D2).

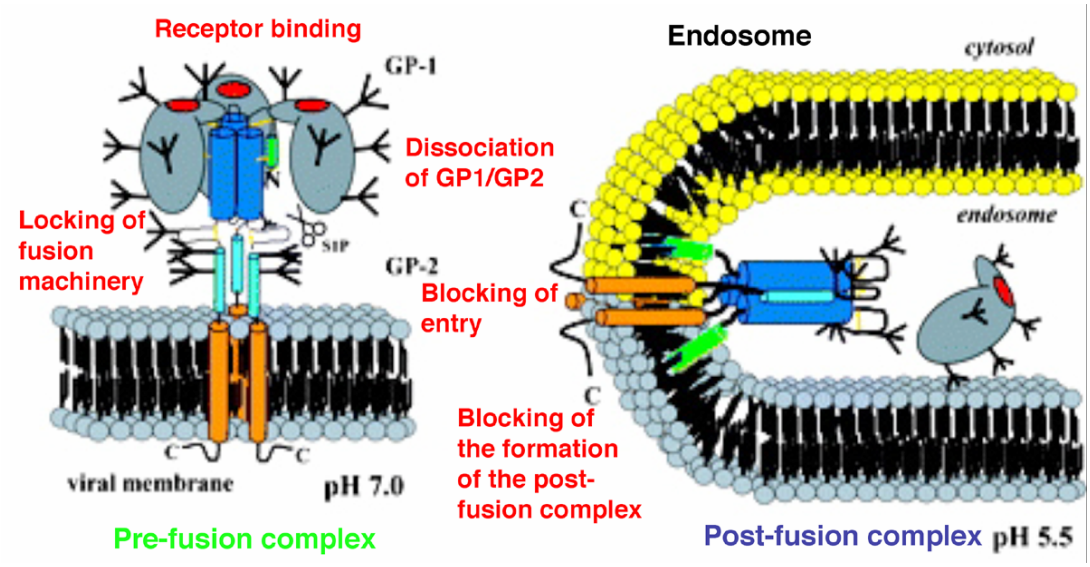

Figure D2: Potential steps in LFVGP-mediated infection that may be blocked by our drugs.

D.4.3 Extension of small molecule screening to other drug targets and other emerging pathogens

Although inhibition of virus-host cell binding and entry is a very promising anti-viral strategy for highly pathogenic viruses, it is a good idea to aim at the development of high throughput screening assays targeting other steps in virus multiplication. One attractive target for inhibitors is the proteolytic processing of LFVGP. We and others have shown that the GPs of the Old World arenaviruses LFV and LCMV are cleaved by the subtilase SKI-1/S1P and that processing of the GP is essential for viral infectivity (Lenz et al., 2001; Beyer et al., 2003; Kunz et al., 2003). Since S1P protease is involved in cholesterol metabolism of the host cell, a temporary block of S1P activity could be compensated by dietary cholesterol supplementation. The S1P protease appears therefore as an attractive drug target to interfere with the processing of LFVGP and thus virus multiplication. Another promising target for interference with viral multiplication is the assembly of LFV virions. Since the presence of the viral

GP is absolutely required for LFV particles to be infectious, GP incorporation during virus assembly at the plasma membrane is another promising target for interference by anti-viral drugs. Based on the incorporation of recombinant LFVGP in our pseudovirion system, one might try to establish an assay system for the screening for small molecule drugs that prevent the formation of mature LFVGP whose then can be incorporated into infections particles.

The identification of candidate small molecule anti-viral drugs against emerging viruses will likely be of enormous benefit for the development of novel anti-viral therapeutics for these severe human pathogens. Furthermore, the elucidation of their mechanism of action will shed light on the molecular mechanism underlying virus-receptor binding, entry, and virus GP-mediated membrane fusion of human pathogenic arenaviruses and thus identify novel drug targets and contribute to subsequent rational drug design.

E. REFERENCES

Andrei, G., and De Clercq, E. (1990). Inhibitory effect of selected antiviral compounds on arenavirus replication in vitro. *Antiviral Res* **14**(4-5)**,** 287-99.

Barresi, R., and Campbell, K. P. (2006). Dystroglycan: from biosynthesis to pathogenesis of human disease. *J Cell Sci.* **119**(Pt 2)**,** 199-207.

Berg, T., Cohen, S. B., Desharnais, J., Sonderegger, C., Maslyar, D. J., Goldberg, J., Boger, D. L., and Vogt, P. K. (2002). Small-molecule antagonists of Myc/Max dimerization inhibit Myc-induced transformation of chicken embryo fibroblasts. *Proc Natl Acad Sci U S A* **99**(6)**,** 3830-5.

Beyer, W. R., Popplau, D., Garten, W., von Laer, D., and Lenz, O. (2003). Endoproteolytic processing of the lymphocytic choriomeningitis virus glycoprotein by the subtilase SKI-1/S1P. *J Virol* **77**(5)**,** 2866-72.

Boger, D. L. (2003). Solution-phase synthesis of combinatorial libraries designed to modulate protein-protein or protein-DNA interactions. *Bioorg Med Chem* **11**(8)**,** 1607-13.

Boger, D. L., Desharnais, J., and Capps, K. (2003). Solution-phase combinatorial libraries: modulating cellular signaling by targeting protein-protein or protein-DNA interactions. *Angew Chem Int Ed Engl* **42**(35)**,** 4138-76.

Boger, D. L., Ducray, P., Chai, W., Jiang, W., and Goldberg, J. (1998). Higher order iminodiacetic acid libraries for probing protein-protein interactions. *Bioorg Med Chem Lett* **8**(17)**,** 2339-44.

Boger, D. L., Goldberg, J., Satho, S., Ambroise, Y., Cohen, S. B., and Vogt, P. K. (2000). Non-amide based combinatorial libraries derived from N-Boc-iminodiacetic acid: solution-pahse synthesis of a 150-membered piperazinone library with activity against LEF-1/beta-catenin mediated transcription. *Helv. Chim. Acta* **83,** 1825-1845.

Boger, D. L., Goldberg, J., Silletti, S., Kessler, T., and Cheresh, D. A. (2001). Identification of a novel class of small-molecule antiangiogenic agents through the screening of combinatorial libraries which function by inhibiting the binding and localization of proteinase MMP2 to integrin alpha(V)beta(3). *J Am Chem Soc* **123**(7)**,** 1280-8.

Borrow, P., and Oldstone, M. B. (1994). Mechanism of lymphocytic choriomeningitis virus entry into cells. *Virology* **198**(1)**,** 1-9.

Buchmeier, M. J., Bowen, M. D., and Peters, C. J. (2001). Arenaviridae: the virus and their replication. Fourth ed. *In* "Fields Virology" (D. M. Knipe, and P. M. Howley, Eds.), Vol. 2, pp. 1635-1668. 2 vols. Lippincott Williams & Wilkins, Philadelphia.

Buchmeier, M. J., Lewicki, H. A., Tomori, O., and Oldstone, M. B. (1981). Monoclonal antibodies to lymphocytic choriomeningitis and pichinde viruses: generation, characterization, and cross-reactivity with other arenaviruses. *Virology* **113**(1)**,** 73-85.

Burns, J. W., and Buchmeier, M. J. (1991). Protein-protein interactions in lymphocytic choriomeningitis virus. *Virology* **183**(2)**,** 620-9.

Candurra, N. A., Maskin, L., and Damonte, E. B. (1996). Inhibition of arenavirus multiplication in vitro by phenotiazines. *Antiviral Res* **31**(3)**,** 149-58.

Cao, W., Henry, M. D., Borrow, P., Yamada, H., Elder, J. H., Ravkov, E. V., Nichol, S. T., Compans, R. W., Campbell, K. P., and Oldstone, M. B. (1998). Identification of alpha-dystroglycan as a receptor for lymphocytic choriomeningitis virus and Lassa fever virus [see comments]. *Science* **282**(5396)**,** 2079-81.

Castilla, V., Larzabal, M., Sgalippa, N. A., Wachsman, M. B., and Coto, C. E. (2005). Antiviral mode of action of a synthetic brassinosteroid against Junin virus replication. *Antiviral Res.* **68**(2)**,** 88-95. Epub 2005 Sep 7.

Cordo, S. M., Candurra, N. A., and Damonte, E. B. (1999). Myristic acid analogs are inhibitors of Junin virus replication. *Microbes Infect* **1**(8)**,** 609-14.

Dutko, F. J., and Oldstone, M. B. (1983). Genomic and biological variation among commonly used lymphocytic choriomeningitis virus strains. *J Gen Virol* **64**(Pt 8)**,** 1689-98.

Elliott, L. H., McCormick, J. B., and Johnson, K. M. (1982). Inactivation of Lassa, Marburg, and Ebola viruses by gamma irradiation. *J Clin Microbiol* **16**(4)**,** 704-8.

Esko, J. D., Stewart, T. E., and Taylor, W. H. (1985). Animal cell mutants defective in glycosaminoglycan biosynthesis. *Proc Natl Acad Sci U S A.* **82**(10)**,** 3197-201.

Fisher-Hoch, S. P., and McCormick, J. B. (2004). Lassa fever vaccine. *Expert Rev Vaccines* **3**(2)**,** 189-97.

Gallaher, W. R., DiSimone, C., and Buchmeier, M. J. (2001). The viral transmembrane superfamily: possible divergence of Arenavirus and Filovirus glycoproteins from a common RNA virus ancestor. *BMC Microbiol* **1**(1)**,** 1.

Garcia, C. C., Candurra, N. A., and Damonte, E. B. (2002). Mode of inactivation of arenaviruses by disulfide-based compounds. *Antiviral Res.* **55**(3)**,** 437-46.

Geisbert, T. W., and Jahrling, P. B. (2004). Exotic emerging viral diseases: progress and challenges. *Nat Med* **10**(12 Suppl)**,** S110-21.

Geisbert, T. W., Jones, S., Fritz, E. A., Shurtleff, A. C., Geisbert, J. B., Liebscher, R., Grolla, A., Stroher, U., Fernando, L., Daddario, K. M., Guttieri, M. C., Mothe, B. R., Larsen, T., Hensley, L. E., Jahrling, P. B., and Feldmann, H. (2005). Development of a new vaccine for the prevention of Lassa fever. *PLoS Med* **2**(6)**,** e183.

Goldberg, J., Jin, Q., Ambroise, Y., Satoh, S., Desharnais, J., Capps, K., and Boger, D. L. (2002). Erythropoietin mimetics derived from solution phase combinatorial libraries. *J Am Chem Soc* **124**(4)**,** 544-55.

Kilby, J. M., and Eron, J. J. (2003). Novel therapies based on mechanisms of HIV-1 cell entry. *N Engl J Med.* **348**(22)**,** 2228-38.

Kunz, S., Edelmann, K. H., de la Torre, J.-C., Gorney, R., and Oldstone, M. B. A. (2003). Mechanisms for lymphocytic choriomeningitis virus glycoprotein cleavage, transport, and incorporation into virions. *Virology* **314** 168-178..

Kunz, S., Sevilla, N., Rojek, J. M., and Oldstone, M. B. (2004). Use of alternative receptors different than alpha-dystroglycan by selected isolates of lymphocytic choriomeningitis virus. *Virology* **325**(2)**,** 432-45.

Lenz, O., ter Meulen, J., Klenk, H. D., Seidah, N. G., and Garten, W. (2001). The Lassa virus glycoprotein precursor GP-C is proteolytically processed by subtilase SKI-1/S1P. *Proc Natl Acad Sci U S A* **98**(22)**,** 12701-5.

McCormick, J. B., and Fisher-Hoch, S. P. (2002). Lassa Fever. *In* "Arenaviruses I" (M. B. Oldstone, Ed.), Vol. 262, pp. 75-110. Springer-Verlag, Berlin, Heidelberg, New York.

McCormick, J. B., King, I. J., Webb, P. A., Scribner, C. L., Craven, R. B., Johnson, K. M., Elliott, L. H., and Belmont-Williams, R. (1986). Lassa fever. Effective therapy with ribavirin. *N Engl J Med* **314**(1)**,** 20-6.

Oldstone, M. B. (2002). Biology and pathogenesis of lymphocytic choriomeningitis virus infection. *In* "Arenaviruses" (M. B. Oldstone, Ed.), Vol. 263, pp. 83-118.

Silletti, S., Kessler, T., Goldberg, J., Boger, D. L., and Cheresh, D. A. (2001). Disruption of matrix metalloproteinase 2 binding to integrin alpha vbeta 3 by an organic molecule inhibits angiogenesis and tumor growth in vivo. *Proc Natl Acad Sci U S A* **98**(1)**,** 119-24.

Spiropoulou, C. F., Kunz, S., Rollin, P. E., Campbell, K. P., and Oldstone, M. B. (2002). New World arenavirus clade C, but not clade A and B viruses, utilizes alpha-dystroglycan as its major receptor. *J Virol* **76**(10)**,** 5140-6.

Stevens, J., Corper, A. L., Basler, C. F., Taubenberger, J. K., Palese, P., and Wilson, I. A. (2004). Structure of the uncleaved human H1 hemagglutinin from the extinct 1918 influenza virus. *Science* **303**(5665)**,** 1866-70.

Tao, Y., Strelkov, S. V., Mesyanzhinov, V. V., and Rossmann, M. G. (1997). Structure of bacteriophage T4 fibritin: a segmented coiled coil and the role of the C-terminal domain. *Structure* **5**(6)**,** 789-98.

Weber, E. L., and Buchmeier, M. J. (1988). Fine mapping of a peptide sequence containing an antigenic site conserved among arenaviruses. *Virology* **164**(1)**,** 30-8.

Yang, Z., Delgado, R., Xu, L., Todd, R. F., Nabel, E. G., Sanchez, A., and Nabel, G. J. (1998). Distinct cellular interactions of secreted and transmembrane Ebola virus glycoproteins. *Science* **279**(5353)**,** 1034-7.